Allergenicity of Camel Milk

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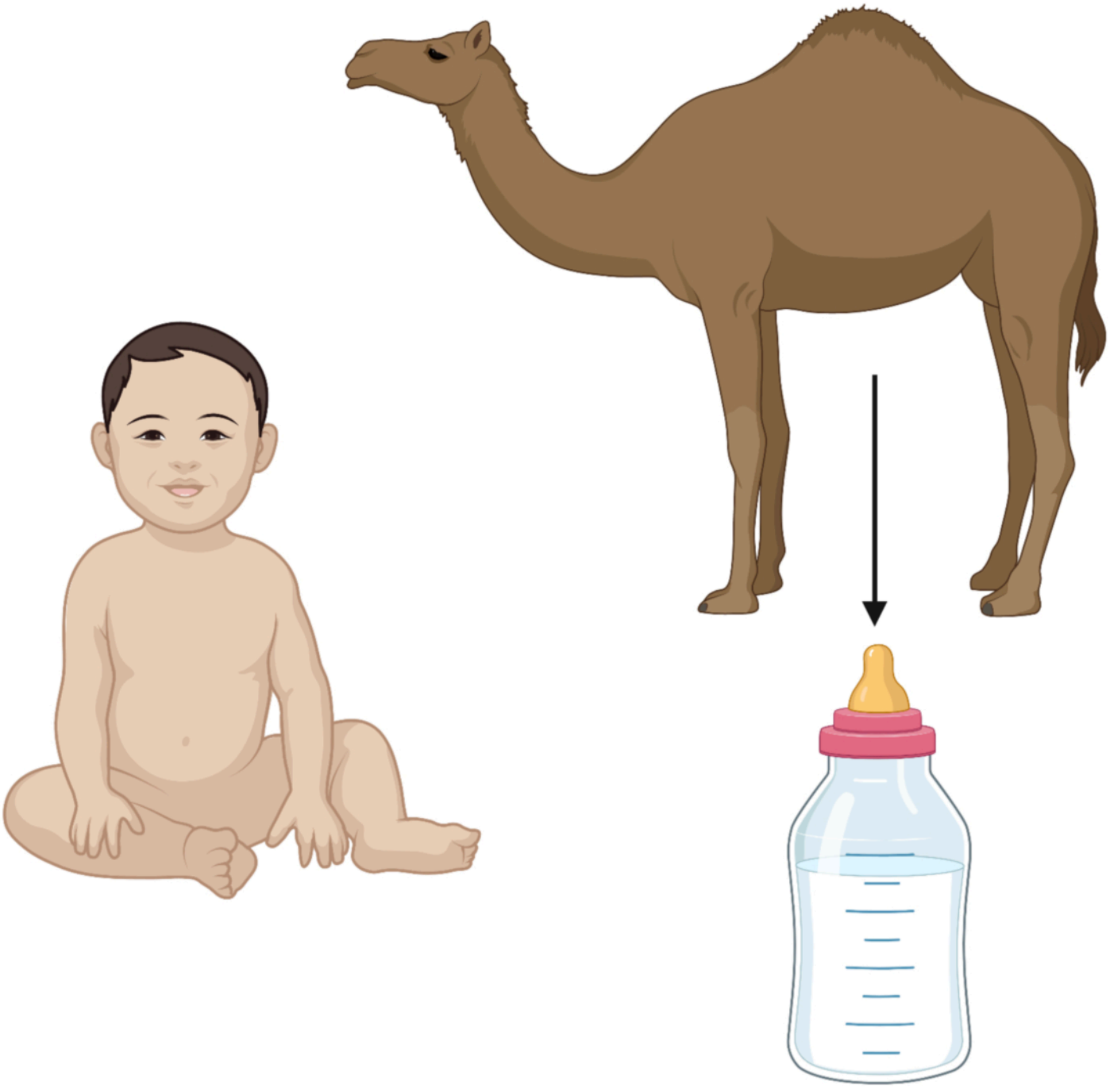

Allergenicity of Camel Milk

PhD Thesis

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National Food Institute

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Preface

This PhD project was performed in the Research Group for Food Allergy at the National Institute, the Technical University of Denmark from May 2018 to June 2022 including eleven months of maternity leave. The project was supervised by Senior Researcher, Head of the Research Group for Food Allergy, Katrine Lindholm Bøgh and co-supervised by Senior Researcher Ana Isabel Sancho from the Research Group for Food Allergy and by Professor Egon Bech Hansen from the Research Group for Gut, Microbes and Health. The project was funded by Ausnutria Dairy (China) Co., Ltd, Changsha, Hunan, China.

Natalia Zofia Maryniak
Kgs. Lyngby, June 2022

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I would like to thank my PhD colleagues present and past from Research Group for Food Allergy. Thanks a lot Anne-Sofie Ravn Ballegaard, Katrine Bækby Graversen, Tiffany Kirkaldy Spaanager Sztuk and Stephanie Ingemann Bisgaard for our continues conversations both about scientific topics and private life. I enjoyed a lot your company through this journey.

Finally, I would like to thank my family and friends. Special thanks to my husband Krzysztof for your endless support, your understanding and patience. Thank you for taking care of our daughter Maja and making me complete this PhD project. Thanks to my parents, my brother, my aunt and my in law family. Without your understanding, care and patience it would not be possible to complete this project. Thanks to all my friends who always listened to and were with me through this time.

Summary

BACKGROUND

Food allergy is a disease which prevalence is increasing over time. There are eight main allergens responsible for food allergy and this are: peanuts, tree nuts, egg, fish, shellfish, cow's milk, soya and wheat. Cow's milk is the main allergen affecting infants and small children. Cow's milk allergy (CMA) affects 0.5-3.8% of all infants and small children and while some outgrow CMA over time, others keep it lifelong. Breastfeeding should always be the first choice for infants feeding. However, sometimes it is not possible or not chosen as a feeding option. When an infant suffers from CMA and cannot be exclusively breastfed, there is a need to use hypoallergenic infant formula.

In this PhD project a literature review was first performed in order to gain an overview on the present and potential, future infant formulas for CMA management and prevention (**Manuscript I**). Alternative sources of proteins in infant formula production are needed due to the climate change and growing population. Plant based proteins gained an interest mainly due to their vegan status and potential lower allergenicity. Mammalian milks such as goat, sheep, donkey, horse and camel have drawn an attention due to their potential of low cross-reactivity with cow's milk proteins. Camel milk is one of mammalian milk gaining an interest for its usability as a protein source in the production of infant formula for CMA management and prevention due to the lack of β-lactoglobulin (BLG) one of the major allergen in cow's milk and also due to the low homology with cow's milk proteins. Yet, there is still a lack of solid scientific evidences on how camel milk is tolerated by cow's milk allergic individuals, how processing such as enzyme hydrolysis and heat treatment influence camel milk proteins immunogenicity, sensitising capacity and cross-reactivity with cow's milk proteins as well as whether camel milk proteins could drive tolerance to cow's milk proteins.

AIMS AND METHODS

The aims of this project were to evaluate (1) immunogenicity, sensitising capacity and cross-reactivity of intact cow's and camel milk (**Manuscript II***), (2) how processing such as enzyme hydrolysis and heat treatment influence cow's and camel milk proteins physicochemical characteristics (**Manuscript III**) and (3) further immunogenicity, sensitising capacity and cross-reactivity of cow's and camel milk processed products (**Manuscript IV**), (4) whether intact camel milk could prevent CMA as well as whether intact cow's milk could prevent camel milk allergy (**Manuscript V**). Finally, the aim of this project was to evaluate (5) how children with CMA reacted towards intact and processed cow's and camel milk (**Manuscript VI**).

Cow's and camel milk proteins physicochemical characteristics before and after processing were evaluated and compared my means of different analytical methods such as electrophoresis and chromatography. Evaluation of immunogenicity, sensitising capacity, cross-reactivity as well as preventive capacity of cow's and camel milk products were evaluated in Brown Norway rat models and further analysed by means of humoral and cellular immune responses. Reactivity

towards cow's and camel milk products in children with confirmed CMA was evaluated by assessing the level antibodies in their serum/plasma samples.

***Manuscript II** includes the results from the animal experiment performed before the beginning of this PhD project. The animal experiment was part of Natalia's master project and most of the samples were analysed during master project duration. However, some analysis were performed and finalised at the beginning of this PhD project. Moreover, whole manuscript was written, submitted and accepted during this PhD project.

RESULTS

Results presented in **Manuscript II** showed that the inherent immunogenicity and sensitising capacity of intact cow's and camel milk was similar. Yet, they showed a low cross-reactivity, being lower between caseins in comparison to whey proteins.

Results presented in **Manuscript III** showed that cow's and camel milk behaved differently under enzyme hydrolysis and heat treatment. For both processing methods differences in protein and other components composition and lack of BLG in camel milk played an important role in inducing distinct modifications between cow's and camel milk.

Results presented in **Manuscript IV** showed that heat treatment and enzyme hydrolysis influenced cow's and camel milk immunogenicity, sensitising capacity, reactivity and its specificity in different ways. In addition heat treatment and enzyme hydrolysis showed to further decrease cross-reactivity between cow's and camel milk proteins.

Results presented in **Manuscript V** showed that camel milk could not prevent CMA while cow's milk showed to have a low and transient capacity to prevent camel milk allergy. Low cross-tolerogenic capacity between cow's and camel milk proteins was suggested to be due to their low protein homology.

Results presented in **Manuscript VI** were in line with results obtained based on animal study in **Manuscript IV** and showed that cow's and camel milk had a low cross-reactivity in children with CMA which could be even decreased by processing methods such as enzyme hydrolysis and perhaps heat treatment.

CONCLUSIONS AND PERSPECTIVES

In conclusion, the results showed that camel milk is a promising source of proteins in infant formula for CMA management. Processing such as enzyme hydrolysis and heat treatment showed to be efficient methods to improve usefulness of camel milk in CMA management. However, the results showed that camel milk is not a good candidate for CMA prevention, due to the lack of cross-tolerance, indicating that if one product is sufficient for CMA management, it would probably not be for CMA prevention.

List of manuscripts

Manuscript I

Maryniak NZ, Sancho AI, Hansen EB, Bøgh KL. Alternatives to Cow's Milk-Based Infant Formulas in the Prevention and Management of Cow's Milk Allergy. Foods. 2022 Mar. 23;11:926. https://doi.org/10.3390/foods11070926.

Manuscript II

Maryniak NZ, Hansen EB, Ballegaard AR, Sancho AI, Bøgh KL. Comparison of the Allergenicity and Immunogenicity of Camel and Cow's Milk - A Study in Brown Norway Rats. Nutrients. 2018 Dec. 4;10(12):1903. https://doi.org/10.3390/nu10121903.

Manuscript III

Maryniak NZ, Sancho AI, Nielsen SD-H, Larsen LB, Gao Y, Bøgh KL, Hansen, EB. Processing induces distinct modifications of cow's and camel milk proteins. *Manuscript in preparation.*

Manuscript IV

Maryniak NZ, Sztuk TKS, Mancino M, Hansen EB, Sancho AI, Bøgh KL. Sensitising capacity of cow's and camel milk products in a Brown Norway rat model – the impact of processing. *Manuscript in preparation.*

Manuscript V

Maryniak NZ, Halkjær MS, Ballegaard ASR, Sancho AI, Hansen EB, Bøgh KL. Camel milk cannot prevent the development of cow's milk allergy – A study in Brown Norway rats. *Manuscript submitted to Molecular Nutrition and Food Research.*

Manuscript VI

Maryniak NZ, Schmidthaler K, Sancho AI, Hansen EB, Szépfalusi Z, Eiwegger T, Bøgh KL. Reactivity towards camel milk products in cow's milk allergic children. *Study report.*

Manuscript not included in the thesis

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Abbreviations

aa	Amino acid
AAF	Amino acid-based infant formula
ALA	α-lactalbumin
Alc	Alcalase
APC	Antigen presenting cell
ARA	Arachidonic acid
AUC	Area under the curve
BAT	Basophil activation test
BCR	B-cell receptor
BLG	β-lactoglobulin
BN	Brown Norway
Bos d	Bos domesticus
B2m	Beta-2 microglobulin
CAS	Casein
cDNA	Complementary deoxyribonucleic acid
CEL	N-ε-(carboxyethyl)lysine
Chtrp	Chymotrypsin
CMA	Cow's milk allergy
CML	bN-ε-(carboxymethyl)lysine
CT	Cholera Toxin
CX3XCR1	C-X3-C motif chemokine receptor 1
DC	Dendritic cells
DHA	Dehydroalanine
DH	Degree of hydrolysis
DIG	Digoxigenin
DTT	Dithiothreitol
EH	Enzyme hydrolysed
eHF	Extensively hydrolysed formula
ELISA	Enzyme-linked immunosorbent assay
EPI	Epithelium
EtOH	Ethanol
EU	Endotoxin units
FoxP3	Forkhead box P3
Glycam-1	Glycosylation-dependent cell adhesion molecule-1
GPC	Gel permeation chromatography
HP	High pressure
HRP	Horse radish peroxidase
HT	Heat treated
IC50	Half minimum inhibitory concentration
I.d.	Intradermal
Ig	Immunoglobulin
I.g.	Intragastric
IL	Interleukin
INF-γ	Interferon-gamma
I.p.	Intraperitoneal
kDa	Kilodalton
KSCN	Potassium thiocyanate
LAL	Lysinoalanine
LAN	Lanthionine
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LF	Lactoferrin
LP	Lamina propria
LOQ	Limit of quantification
mLN	Mesenteric Lymph Noode
MRM	Multiple Reaction Monitoring
MW	Molecular weight
NA	Not available
Ocln	Ocludin
OD	Optical density
OFC	Oral food challenge
OPA	O-phthalaldehyde
OVA	Ovalbumin
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-tween
pHF	Partially hydrolysed formula
PP	Peyer's patches
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RT	Room temperature
SA	Serum albumin
SD	Standard deviation
SDHA	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SI	Small intestine
SPT	Skin prick test
TCR	T-cell receptor
TGF-β	Transforming Growth Factor-β
TMB	3, 3', 5, 5'-tetramethylbenzidine
Trp	Trypsin
TSLP	Thymic stromal lymphopoietin
UHT	Ultra-high temperature
UV	Ultraviolet
v/v	Volume/volume
WAP	Whey acidic protein
w/v	Weight/volume
w/w	Weight/weight

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Manuscript I

Alternatives to Cow's Milk-Based Infant formulas in the Prevention and Management of Cow's Milk Allergy

Maryniak NZ, Sancho AI, Hansen EB, Bøgh KL.

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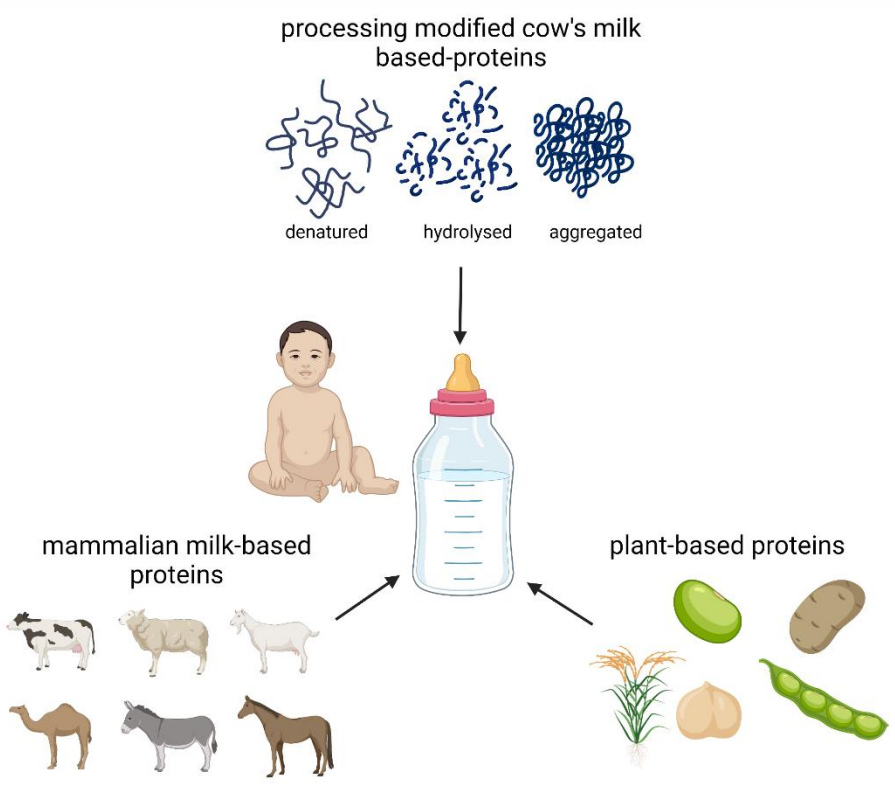

Graphical abstract

foods

MDPI

Review

Alternatives to Cow's Milk-Based Infant Formulas in the Prevention and Management of Cow's Milk Allergy

Natalia Zofia Maryniak, Ana Isabel Sancho, Egon Bech Hansen and Katrine Lindholm Bøgh *

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Abstract: Cow's milk-based infant formulas are the most common substitute to mother's milk in infancy when breastfeeding is impossible or insufficient, as cow's milk is a globally available source of mammalian proteins with high nutritional value. However, cow's milk allergy (CMA) is the most prevalent type of food allergy among infants, affecting up to 3.8% of small children. Hypoallergenic infant formulas based on hydrolysed cow's milk proteins are commercially available for the management of CMA. Yet, there is a growing demand for more options for infant feeding, both in general but especially for the prevention and management of CMA. Milk from other mammalian sources than the cow, such as goat, sheep, camel, donkey, and horse, has received some attention in the last decade due to the different protein composition profile and protein amino acid sequences, resulting in a potentially low cross-reactivity with cow's milk proteins. Recently, proteins from plant sources, such as potato, lentil, chickpeas, quinoa, in addition to soy and rice, have gained increased interest due to their climate friendly and vegan status as well as potential lower allergenicity. In this review, we provide an overview of current and potential future infant formulas and their relevance in CMA prevention and management.

Keywords: infant formula; processing; plant-based proteins; mammalian milk-based proteins; alternative infant formula; cow's milk allergy; allergy prevention; allergy management

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1. Introduction

Mother's milk is constantly changing to adapt to the need of the infant as the infant grows. The composition and nutrients, including proteins, carbohydrates, vitamins, hormones, antibodies, antibacterial agents, growth factors, and cytokines, change according to the infants age for proper development and immune modulation [1]. In contrast, formulas are divided into stage one infant formulas (0–6 months of age), stage two follow-up formulas (6–12 months of age), and stage three toddler formulas (above 12 months of age) to adapt to the need of infants at different stages of development. Breastfeeding, in comparison to use of infant formulas, provides many benefits, such as better brain development and protection against infections as well as obesity [1–3]. In general, it is recommended to breastfeed for at least the first 6 months of the infant's life, further continuing the breastfeeding while introducing complementary foods [4]. Infant formulas are specific products produced as a substitute to mother's milk for situations where breastfeeding is not possible or is insufficient. They are required to fulfil certain nutritional requirements [5] and are mainly based on cow's milk proteins. When an infant has been diagnosed with cow's milk allergy (CMA) and cannot be fully breastfed, the use of a hypoallergenic cow's milk-based, extensively hydrolysed formula (eHF) is generally recommended for management of the CMA, with amino acid-based infant formula (AAF) as an alternative if the cow's milk-allergic infants suffer from severe CMA or cannot tolerate the eHF. Infant formulas based on plant proteins are in some countries recommended as a second choice for the management of CMA [1].

At present, in EU, infant formulas can only be based on cow's and goat milk proteins, soy proteins, as well as hydrolysed proteins [6,7]. Yet, infant formulas based on alternative process-modified versions of cow's milk proteins, from other mammalian milk, or based on other plant proteins have been suggested and investigated for various reasons. One main interest in providing new and alternative infant formulas is for use in the prevention and management of CMA, as infants suffering from CMA cannot tolerate conventional cow's milk-based infant formulas and, in some situations, may not even tolerate eHFs [8]. Another reason to search for alternative infant formulas is the increasing interest in plant-based diets connected to environmental, climate, and ethical issues [9,10]. In the present review, we will provide an overview and discuss current and potential future options for infant formulas in the context of CMA prevention and management.

2. Food Allergy

Food allergy, which is defined as an immune-mediated adverse reaction to otherwise harmless food proteins [11], is a growing global health problem [12]. More than 70 foods have been reported to induce allergic reactions after their consumption, and eight of them are responsible for more than 90% of all reactions [13,14]. These are peanut, tree nut, cow's milk, soy, wheat, hen's egg, fish, and shellfish [14].

Food allergy affects ~1–3% of adults and ~6–8% of small children although the reported prevalence seems to differ between individual studies, countries, and continents [13,15,16]. The prevalence is observed to be higher in small children than in adults because many children naturally outgrow their food allergy over time, gaining tolerance to foods they were previously allergic to [17,18]. There is no unequivocal explanation on why some children outgrow their food allergy while others do not, but several host-, environmental- and allergen-relating factors may be contributing determinants, such as disease severity, gut immune system maturation, gut microbiota composition, type(s) and numbers of culprit allergen(s), or epitope recognition pattern [19–22].

At present, there are only very limited treatment possibilities, and strict avoidance of the offending foods is the main viable management option [23]. While food allergy immunotherapy is generally considered an experimental treatment, with many ongoing studies investigating different routes of administration, dosing regimens, as well as efficacy and safety [24–26], one oral immunotherapy for peanut allergy has been approved by Food and Drug Administration (FDA) [27]. Proper education is an important factor in food allergy management in order to guide patients' attention to food labelling and their correct interpretation [28,29], raise awareness of possible cross-reactions with other food products [29,30], as well as for patients to know when and how to use prescribed medication [31]. Food allergy may have a negative impact on life quality [32], especially for kids who report decreased quality of social life and increased anxiety [33,34].

Based on the mechanisms behind food allergy, the disease can be classified as either IgE-mediated or non-IgE-mediated allergy [35]. IgE-mediated food allergy is the best known and characterised type of food allergy [35,36] and can be divided into two phases: a sensitisation phase and an elicitation phase [37]. Upon a first exposure to food proteins, sensitisation may occur, when the immune system is introduced to the antigens for the first time. Antigen presenting cells (APCs), mostly dendritic cells (DCs), take up the food proteins or fragments hereof and process them into smaller peptides, which they present on their surface MHC II molecules to T-cell receptors (TCRs) on naïve T cells specific for the particular peptide. T cells are activated upon further signalling events with ligation of CD28 on the naïve T cells with CD80 and CD86 expressed on the surface of DCs as well as with co-stimulatory signals from pro-inflammatory cytokines IL-4, IL-25, IL-33, and TSLP [38,39], which causes the naïve T cells to differentiate into CD4+ Th2 cells [40,41]. Activated and differentiated Th2 cells interact with naïve B cells through their TCRs and allergen bound to MHC II on naïve B cells as well as through signalling events provided by binding of CD40L on the Th2 cells with CD40 on the B cells. This together with co-stimulatory signals from IL-4 and IL-13, secreted by Th2 cells, cause the B cells to maturate and differentiate into food

allergen-specific IgE-secreting plasma cells [37,42]. Secreted food allergen-specific IgE binds to the high-affinity FcεRI receptors on the surface of tissue mast cells or blood basophils [37], which completes the sensitisation phase (Figure 1). The elicitation phase takes place upon subsequent exposures to the same or cross-reactive food allergens, where the allergens cross-link FcεRI-bound allergen-specific IgEs on the surface of the mast cells and basophils leading to their degranulation and release of mediators, such as histamine [37] (Figure 1). These mediators are responsible for the symptoms characterising the food allergic reaction, which can involve many organs causing, e.g., gastrointestinal disorders, respiratory tract inflammation, skin and eye itching and swelling, and in worst cases, life-threatening anaphylaxis [13].

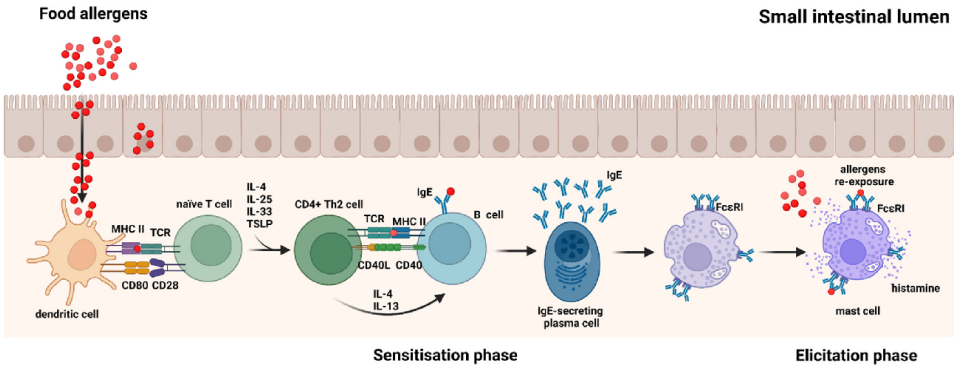

Figure 1. Mechanisms of IgE-mediated food allergy. IgE-mediated food allergy is divided into two phases: a sensitisation and an elicitation phase. In the sensitisation phase, food allergens are taken up by dendritic cells, which process allergens into smaller peptides and present them on MHC II molecules to T-cell receptors (TCRs) on naïve T cells. T cells are activated upon ligation of CD28 on the surface of naïve T cells and CD80 on the surface of dendritic cells, with co-stimulation from pro-inflammatory cytokines IL-4, IL-25, IL-33, and TSLP. Activated and differentiated Th2 cells interact and activate naïve B cells through TCR and antigen bound to MHC II on naïve B cells as well as through ligation of CD40L on the surface of Th2 cells and CD40 on the surface of B cells, together with co-stimulation from IL-4 and IL-13 for maturation and differentiation of B cells into food allergen-specific IgE-secreting plasma cells. Secreted food allergen-specific IgEs bind to high-affinity FcεRI receptors on tissue mast cells and/or blood basophils. In the elicitation phase, re-exposure to the same or cross-reactive food allergens causes allergen cross-linking of FcεRI-bound specific IgEs on the surface of tissue mast cells and/or blood basophils leading to their degranulation and release of mediators, such as histamine. Graphics created with BioRender.com.

2.1. Cow's Milk Allergy

IgE-mediated CMA is the most common food allergy among infants and small children, affecting between 0.5 and 3.8% of the children [15,43,44]. Fortunately, most children outgrow their CMA, acquiring tolerance to cow's milk [45], though some keep it for life [18]. CMA is usually one of the first food allergies diagnosed in infants, as cow's milk proteins are often the first food proteins introduced to infants due to their presence in infant formulas [46]. Symptoms of IgE-mediated CMA most often appear immediately, within few minutes after consumption of the cow's milk-based dairy product [47], and may reveal as diarrhoea, vomiting, skin itching, urticaria, or breathing problems, and may potentially cause anaphylaxis that can be fatal [48].

Little is known on why some individuals develop tolerance after consumption of cow's milk proteins, while others develop an abnormal immune response towards the proteins. However, CMA can to some degree be "inherited", as the atopy status of the child's parents and siblings may be predictive for the risk of developing CMA [49].

Cow's milk contains ~32 g of proteins per litre [50], which are divided into two protein fractions: caseins that represent ~80% and whey proteins that represent ~20% of the total proteins (Table 1) [51,52].

Table 1. Cow's milk allergens and their characteristics. Table modified from [53].

Cow's Milk Fraction	Protein	Allergen Name	Content (%)	Size (kDa)	Major/Minor Allergen	S-S Bridges
Casein (80%)	α_{s1}-casein	Bos d 9	32	23.6	Major	0
	α_{s2}-casein	Bos d 10	10	25.2	Major	0
	β-casein	Bos d 11	28	24	Major	0
	k-casein	Bos d 12	10	19	Major	1
Whey (20%)	α-lactalbumin	Bos d 4	5	14.2	Major	4
	β-lactoglobulin	Bos d 5	10	18.3	Major	2 + 1 free
	Serum albumin	Bos d 6	1	66.3	Minor	17 + 1 free
	Immunoglobulins	Bos d 7	3	160	Minor	number varies [1]
	Lactoferrin		<1	80	Minor	16

[1] The number of disulphide (S-S) bridges in immunoglobulins varies depending on their classes as well as subclasses [54].

Cow's milk allergens are designated Bos d, based on the three first letters of the genus and the first letter of the species epithet (*Bos domesticus*), followed by an identification number [55]. Bos d 8 is the allergen name registered in AllergenNomenclature covering all caseins [56]. However, as caseins are divided into four distinct types, they also have specific allergen names, with Bos d 9 designating α_{s1}-casein, Bos d 10 designating α_{s2}-casein, Bos d 11 designating β-casein, and Bos d 12 designating κ-casein. α_{s1}-casein is the most abundant casein found in cow's milk, comprising ~32%, followed by β-casein comprising ~28%, α_{s2}-casein comprising ~10%, and κ-casein comprising ~10% (Table 1). They are classified as secreted calcium-binding phosphoproteins [57] with a loose tertiary structure. In their soluble form, they create quaternary structures called casein micelles. Casein micelles contain a hydrophobic core consisting of α_{s1}-casein, α_{s2}-casein, and β-casein interacting with calcium phosphate and a hydrophilic surface layer of κ-casein [58]. In general, the casein micelle structure is dynamic and changes with factors such as pH, temperature, and pressure. For example, under rennet treatment, casein micelles lose their solubility and precipitate forming aggregates [59], and at various temperatures, micelles may form numerous interactions to a different extent with whey proteins and other milk components [60]. Caseins are all major allergens considered to be involved in more than 50% of all IgE-mediated CMA reported cases [61].

The most abundant whey protein is β-lactoglobulin, designated Bos d 5, which represents ~10% of total proteins in cow's milk and is followed by α-lactalbumin, designated Bos d 4, comprising ~5%; immunoglobulins, designated Bos d 7, comprising ~3%; bovine serum albumin, designated Bos d 6, comprising ~1%; and lactoferrin, comprising <1% (Table 1). β-lactoglobulin and α-lactalbumin are considered major allergens from the whey fraction. They are globular proteins, stabilised by disulphide bridges [62]. Even though bovine serum albumin is found in cow's milk in only low quantities, it is also a common allergen, as up to 50% of cow's milk allergic patients develop IgE specific for this protein [63,64]. Together with lactoferrin, bovine serum albumin is characterised by a high number of disulphide bridges (Table 1), making their tertiary structure highly stable even under denaturing conditions [64]. Lactoferrin is a protein not registered as an allergen in the AllergenNomenclature [56], however, human and animal experimental studies showed their ability to induce allergic reactions [65–67].

Generally, it is not so common that cow's milk allergic patients react to only one cow's milk protein, as CMA is usually characterised by reactivity to multiple cow's milk allergens, including both the unstructured caseins and the globular whey proteins [67]. There are

specific sites within the protein sequence and/or structure that IgEs bind to, which are called epitopes [68]. These epitopes can be either linear or conformational, with linear epitopes consisting of a continuous amino acid sequence of the primary protein structure and conformational epitopes consisting of discontinuous amino acid sequences brought together by the secondary, tertiary, and quaternary folding of the protein [68,69]. Both types of epitopes are found in cow's milk allergens [37,70].

2.2. *Prevention and Management of Cow's Milk Allergy*

To prevent the development of CMA in high-risk infants as well as to manage CMA to avoid elicitation of allergic reactions in already allergic infants, guidelines have been devised providing specific recommendation. Yet, recommendations for CMA prevention and management have changed during the past decade, as recent studies have provided new knowledge with further evidence on for example maternal elimination diet [71], vaginal birth versus caesarean [72,73], pre- and probiotics supplementation [74,75], duration of breastfeeding [76], time of introduction of allergenic foods [77–79], and use of hydrolysed infant formulas [76,80] in relation to CMA prevention and management.

The European Academy of Allergy and Clinical Immunology (EAACI) guidelines on CMA prevention published in 2014 and 2021 concluded that there is no need for maternal elimination diet during pregnancy as well as during lactation period, as the majority of trials have shown no relationship between maternal elimination diet and a reduction in the probability of CMA occurrence in offspring [23,81]. These conclusions provided by the EAACI guidelines for CMA prevention are in line with the Australasian Society of Clinical Immunology and Allergy (ASCIA) guidelines for food allergy prevention from 2005 and 2019 [82,83] as well as with the guideline by the American Academy of Allergy, Asthma, and Immunology (AAAAI) on CMA prevention from 2021 [84]. In addition, a Cochrane Systemic Review by Kramer and Karkuma based on five trials concluded that there is no relation between elimination diet during pregnancy and lactation and a lower likelihood of events of atopic diseases [85]. On the other hand, a cohort study by Tuokkola et al. showed that consumption of cow's milk proteins during pregnancy and lactation contributed to a lower risk of CMA development in offspring compared to those whose mothers avoided the consumption of cow's milk proteins during pregnancy and lactation [86]. This is in line with a study by Stravik et al., which showed similar results [87].

Transmission of the maternal microbiome during vaginal birth is a very important and beneficial factor influencing later gut microbiota development in the infant [88]. The first 1000 days of a child's life is crucial for lifelong gut microbiota shaping [89]. Gut microbiota composition is known to have an influence on many health aspects, including probability of development of many diseases [90]. In relation to CMA prevention, the evidence is contradictory, as some studies have shown no relationship between caesarean delivery and thus the lack of maternal vaginal microbiome transmission and an increased risk of developing allergy [73,91], while others reported such relationship, indicating a beneficial impact of maternal microbiome transmission during vaginal birth for prevention of CMA in offspring [92,93].

Supplementation of probiotics may in some situations be beneficial for an infant. This has, for example, been shown during antibiotic treatment, where the gut microbiota can be heavily disrupted [94]. However, from the perspective of CMA prevention, there is no evidence for or against the use of probiotics in both infants as well as in the breastfeeding mothers [95,96]. For prebiotics, such as galacto-oligosasccharides (GOS) and fructo-oligosaccharides (FOS), which are used for infants to promote a healthy gut microbiota, there is also no evidence for or against their use in the prevention of CMA [75].

In relation to breastfeeding as a potential factor in preventing CMA, current evidence shows no relation between breastfeeding and lower risk of CMA development. However, breastfeeding is anyway strongly recommended by EAACI [81], ASCIA [83], and AAAAI [84] guidelines, as it has many benefits for both infant and mother. Therefore, it should always be the first choice for infant feeding, as mother's milk has the best nutritional

composition designed to meet the infant's need, as it changes continuously, adapting to infants' specific age and hence growth need. Furthermore, the World Health Organisation (WHO) strongly recommends exclusive breastfeeding for the first 6 months of life and thereafter continued breastfeeding while introducing complementary food for as long as the child and mother are willing to [97].

Delayed introduction of the most common allergens by time of complementary food introduction for prevention of food allergy, including CMA, is strongly discouraged by EAACI, ASCIA, and AAAAI, as there is no evidences for its beneficial effect [81,83,84]. In fact, several studies have shown that early introduction of allergenic foods, such as peanuts [98,99], cow's milk [100,101], or hen's egg [99], can decrease the risk of developing food allergy against the particular allergens [102].

Recommendations on the use of special infant formulas based on hydrolysed cow's milk proteins for CMA prevention have changed over the past years. Until recently, it was recommended to use a cow's milk-based partially hydrolysed formula (pHF) for infants in high-risk of developing CMA [23,82]. However, as the current evidence shows no relationship between the use of pHF and a decreased risk of developing CMA [103], the recent guidelines from EAACI, ASCIA, and AAAAI do not recommend using pHF or any other specific infant formulas for CMA prevention [81,83,84].

There are several guidelines with recommendations for CMA management available. The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) [104], British Society for Allergy and Clinical Immunology (BSACI) [105], as well as World Allergy Organisation (WAO) [106] created practical guidelines for CMA diagnosis and management, which are consistent. For the management of CMA in infants, breastfeeding along with strict avoidance of intact cow's milk proteins are the first strategies recommended [104–107]. If breastfeeding is impossible or insufficient, the use of a hypoallergenic infant formula is recommended with eHF as a first choice [104,105,108], where a hypoallergenic infant formula is required to be tolerated by at least 90% of the infants with confirmed CMA, with a confidence interval of 95% in a clinical cohort [80]. If clinical symptoms occur with the use of eHF, the use of AAF is the recommended as the second choice for CMA management [104,105,108].

It has been reported that ~0.5% of infants exclusively breastfed develop cow's milk allergic reactions though mostly reported as being mild or moderate [108]. This may be due to low quantities of cow's milk proteins being present in breastmilk after the consumption of dairy products by the mother [109]. Thus, the maternal diet while breastfeeding an infant diagnosed with CMA needs to be monitored by physician, and in most cases, elimination of any products containing cow's milk proteins is recommended for the mother [104,110]. Another important factor for CMA management is awareness of possible cross-reactivity between cow's milk proteins and proteins from other mammalian milks. In addition, proteins found in cow's milk can also be found elsewhere than in milk as, for example, serum albumin, a whey protein, is also present in beef meat as well as in cow's dander [111].

3. Infant Formulas

Breastfeeding is not always a possibility, as it may be insufficient or not chosen for several reasons. Hence, an alternative to breastfeeding is needed.

Infant formulas are substitutes to breast milk, manufactured in order to fulfil the nutritional requirements of infants allowing their ordinary growth [112,113]. They should mimic breast milk, providing similar conditions for infants' development before and during the introduction of complementary food, until the complete transition to solid food [51]. Indeed, in EU, infant formulas are strictly regulated and need to comply with the Regulation EU 2016/127 with regards to specific compositional and informational requirements [114]. This EU legislation incorporates the principles from WHO Code of Breastmilk Substitutes [115]. If an infant is not breastfed, formulas should be the main source of nutrition for the infant up to 12 months of age. In the EU, the only sources of protein allowed in infant and follow-up formulas are cow's milk, goat milk, soy, as

well as hydrolysed proteins [7]. Yet, the major part of infant formulas are based on cow's milk proteins and should not be confused with any unmodified, raw, or pasteurised milk commercially available [116], as these are not able to fulfil the nutritional requirements of the infants [5,117]. Infant formulas, in general, contain a higher amount of protein compared to breast milk but a lower amount of protein compared to regular cow's milk [5]. In addition, the protein composition may differ, with the proteins in soy-based formulas being very different from those in breast milk [118], and where, for example, the ratio of casein to whey proteins present in cow's milk-based infant formulas may differ from the ratio in breast milk as well as in regular cow's milk, which may influence the properties of infant formulas, including their digestibility [5,119]. A slower digestion kinetics of casein-dominant infant formulas compared to whey-dominant formulas have been shown using an in vitro dynamic infant gastric simulator, which might be explained by a greater extent of aggregations in the casein-based formulas [120].

The lipid content in infant formulas is designed to mimic the composition and amount in breast milk and consists of long-chain polyunsaturated fatty acids (LCPUFAs), such as eicosapentaenoic acid (EPA) [121] and docosahexaenoic acid (DHA), for proper brain development [122] as well as arachidonic acid (ARA) for proper nervous system and muscles development [123]. As the lipid composition in infant formulas should mimic the composition in human milk as much as possible [124], human milk oligosaccharides (HMOs) have gained an increasing interest in the recent decade, especially due to their important impact on the development of a healthy gut microbiota and immune system [125]. GOS and FOS are often included in infant formulas as prebiotics for proper intestinal microbiota development [126]. Iron is an important mineral for a proper neurodevelopment, for which reason, in contrast to regular cow's milk, infant formulas are fortified with iron [127,128].

4. Cow's Milk-Based Infant Formulas

Most infant formulas commercially available are based on cow's milk proteins [51] due to the great availability of the dairy cow's milk worldwide, which corresponds to 81% of the worlds' milk production [129]. In this review, "infant formula" refers to cow's milk-based infant formulas unless stated otherwise. In general, infant formula manufacture is based on milk reconstitution, where different milk fractions, including proteins (whey proteins and/or caseins), fat, and micro- and macronutrients together with other non-milk-based ingredients, are mixed together in specific quantities to fulfil infant formula standards and nutritional requirements in accordance with Regulation EU 2016/127 [114].

Infant formulas can be sold as powder, liquid concentrate, or ready-to-use liquid, where powder-based infant formulas are the cheapest and most common [130]. Figure 2 displays infant formula powder production steps, where technological processes, including pasteurisation, homogenisation, fractionation, heat treatment, mixing, emulsification, evaporation, spray drying, and packaging, are used [51,130,131]. Infant formulas can be based on the whey or casein fraction or both [132]. If both fractions are used in infant formula, fractionation is still applied for whey and caseins separation for their further ratio adjustment (Figure 2). More infant formulas based on whey proteins than caseins are available due to the focus on optimal utilisation of whey after cheese production [133]. There are two methods for mixing additives with milk proteins that can be applied during powdered infant formula manufacture: wet or dry mixing (Figure 2) [130]. In the wet mixing method, additives in liquid form are added to liquid milk proteins, and subsequently, they are spray dried together, whereas in the dry mixing method, additives are added after spray drying to powdered milk proteins, and powders are mixed together. Infant formulas, where no additional processing steps are applied for intentional induction of changes in protein structures, are known as conventional infant formulas. These formulas, based on intact cow's milk proteins, are the most widely applied formulas but are not recommended for infants with CMA [106,134]. Infant formulas that are produced to be

used for CMA management are based on enzyme hydrolysed cow's milk proteins, where enzyme hydrolysis is applied after protein fractionation (Figure 2) [135].

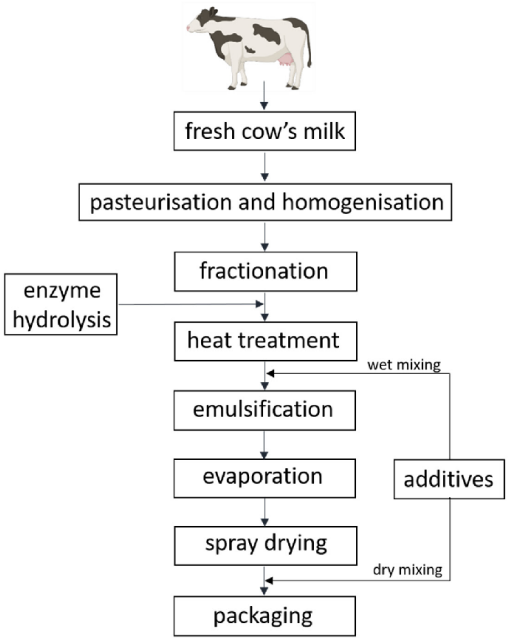

Figure 2. Example of main steps involved in the manufacturing of powdered infant formula. Fresh cow's milk is at first standardised by means of pasteurisation and homogenisation. Further, milk proteins are going through fractionation, heat treatment, mixing, emulsification, evaporation, spray drying, and packaging. Additives are added and mixed with the protein fraction by means of wet or dry mixing. If wet mixing is applied, additives are added in a liquid form to a liquid protein fraction and together undergo emulsification, evaporation, spray drying, and finally infant formula powder packaging. If dry mixing is applied, the same steps are applied with the exception of additives being mixed with proteins in powdered form after spray drying. Additionally, if an infant formula is produced to be used in CMA management, enzyme hydrolysis of proteins is applied after the fractionation step. Picture of the cow is from BioRender.com.

4.1. Reduction of Cow's Milk Protein Allergenicity by Process Modifications

The most common alternatives to conventional infant formulas are infant formulas based on cow's milk proteins, where the proteins are altered to a degree that allows a decrease in their allergenicity, still keeping their nutritional, functional, and palatable properties [136].

Alteration and hence potential reduction of cow's milk protein allergenicity may, in general, be induced by several processing technologies, such as enzymatic hydrolysis, fermentation, heat treatment, high pressure (HP), and radiation (Figure 3). The overall aim of such processing is to diminish or even destroy the IgE-binding epitopes in order to avoid de novo sensitisation in an infant not previously exposed to cow's milk proteins or to avoid cross-linking of IgEs on the surface of tissue mast cells and blood basophils, averting degranulation and hence elicitation of an allergic response in CMA infants [134,137]. Reduction and/or destruction of the IgE-binding epitopes are caused by protein aggregation, denaturation, and degradation. Hence, it is also well recognised that general cooking

alters food protein allergenicity by changing, masking, or even destroying IgE-binding epitopes [134].

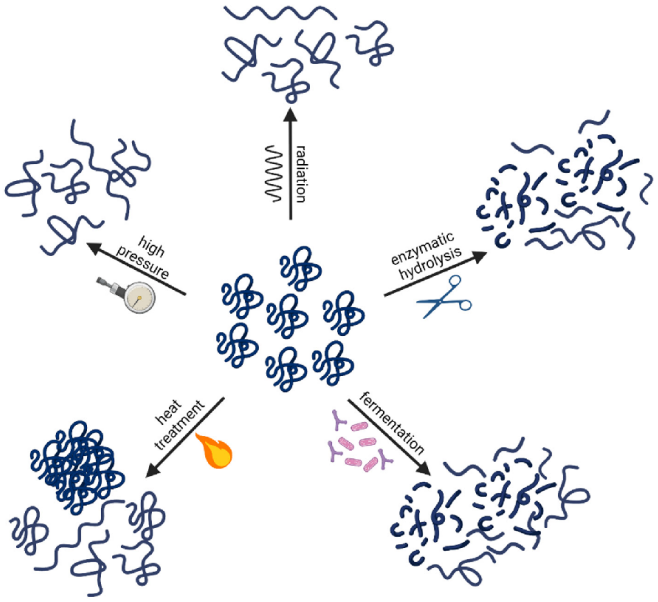

Figure 3. Common protein structural modifications induced by different processing technologies. Enzyme hydrolysis and fermentation may lead to proteolysis of the proteins, destroying primary, secondary, tertiary, as well as potential quaternary structures and causing proteins to break down to smaller peptides. Heat treatment may cause protein denaturation and/or aggregation, while high pressure and radiation may cause protein denaturation. Graphics created with BioRender.com.

4.1.1. Enzymatic Hydrolysis

Enzyme hydrolysis is the most common process used in the infant formula industry to induce protein modifications. The main purpose of this process is to break linear as well as conformational epitopes, hence destroying primary, secondary, tertiary, as well as potential quaternary protein structures (Figure 3) [8]. Enzyme hydrolysis can result in different degree of hydrolysis (DH) depending on the number of enzymes used, their specificity, as well as the conditions applied, such as pH, duration, and temperature. The most common enzymes used in the infant formula industry are recombinant, non-porcine-based proteases for final product Kosher and Halal status [138]. There is a great variation in susceptibility to hydrolysis between different cow's milk proteins depending on their structure and the enzyme(s) used. In general, whey proteins, and especially β-lactoglobulin, are known to be more resistant to proteolysis than caseins [53]. This is explained by the globular structure of the whey proteins that is stabilised by a number of disulphide bridges (Table 1), making it difficult for enzymes to access their cleavage sites. It is, however, shown that susceptibility of proteins to proteolysis can be increased with a preceding heat treatment for protein unfolding [139].

Infant formulas based on hydrolysed cow's milk proteins can be classified as either eHF or pHF depending on the sizes of the peptides in the final product relating to the DH [140]. There is no uniform definition of eHF and pHF [80,141], but in general, eHFs predominantly contain peptides of sizes below 3 kilodalton (kDa), whereas pHFs predominantly contain peptides of sizes below 5 kDa although larger peptides may appear [80,141]. However, great

variations exist between different products, and even significant product batch-to-batch variations have been demonstrated [8,132].

In general, there is no universal definition of a hypoallergenic infant formula. Yet, infants with confirmed CMA are recommended the use of a hypoallergenic infant formula, where the allergenicity of proteins are reduced in order to avoid elicitation of allergic reactions, thus suitable for the management of CMA [104]. The American Academy of Pediatrics (AAP) described requirements that an infant formula needs to fulfil in order to be used for CMA management, thus having "hypoallergenic" status [80]. The hypoallergenic infant formulas should be tolerated by at least 90% of the infants with confirmed allergy to cow's milk proteins with a confidence interval of 95% based on clinical trials [6,80,135]. eHFs are produced to meet these requirements, and according to ESPHGAN [104], all peptides in eHFs should have a size < 3 kDa and be dominated by peptides with a size ~1.5 kDa, hence containing at maximum one linear epitope and thus should not be able to cross-link IgEs on the surface of tissue mast cells and blood basophils and cause allergic reactions [1,142]. eHFs are recommended as a first choice for infant CMA management when breastfeeding is insufficient, impossible, or simply not chosen [104,143]. Yet, some infants with CMA experience allergic reactions, even anaphylaxis, upon feeding with eHFs [104] and consequently may rely on AAF (see Section 5) or if available a soy- or hydrolysed rice-based formula [144] (see Sections 7.1 and 7.2).

pHFs are characterised by their reduced allergenicity compared to conventional infant formulas [145], providing them with decreased potency to induce de novo sensitisation. Yet, they still contain peptides large enough to be recognised by the immune system for induction of tolerance, hence maintaining the tolerogenic properties [141]. However, infant formulas containing peptides between 3–5 kDa, thus being composed of 22–36 amino acids, may induce an allergic reaction, as the peptides could potentially contain two IgE-binding epitopes, allowing for cross-linking of IgEs on the surface of tissue mast cells or blood basophils [1].

Allergenicity as well as eliciting capacity of pHF were evaluated in human studies. For example, Niggemann el al. showed a reduced allergenicity of pHF in patients with CMA as well as reduced eliciting capacity based on skin prick test (SPT) [146], while a study by Caffarelli et al. showed reduced allergenicity and reduced eliciting capacity of some pHFs but not of other pHFs when compared to cow's milk [147]. Moreover, there are animal models established for the evaluation of inherent immunogenicity and allergenicity of infant formulas as well as for the assessment of their preventive capacity [148–152]. Several animal studies have shown reduced allergenicity of pHF [153–155]. In one study, it was shown that pHFs did not induce sensitisation [155], while another study showed induction of sensitisation but without clinical symptom manifestation [156].

Several human studies have been conducted for evaluation of the preventive effect of pHF on CMA development, showing different outcomes. Whereas Vandenplas et al., Chandra, and van Berg et al. provided evidence for a preventive effect of pHF on CMA development when comparing with conventional infant formula [157–159], Lowe et al. did not find evidence to support use of pHF in CMA prevention in comparison to conventional infant formula [160]. In agreement with Lowe et al., a systemic review by de Silva et al. concluded that neither the use of hydrolysed infant formula nor avoidance of conventional infant formula had an effect on CMA prevention [161]. Results from animal studies are in line with the results from human studies, with some studies showing a preventive effect of pHFs on CMA development, while other studies did not show such effect. For example, studies by Graversen et al., Jensen et al., and Fritsche et al. showed that partially hydrolysed whey had a capacity to induce oral tolerance to intact whey proteins [151,162,163]. Further, Chikhi et al. showed that partially hydrolysed whey induced a partial prevention of sensitisation to β-lactoglobulin but no prevention of sensitisation to caseins [164].

Differences in the results from both animal and human studies in relation to the preventive effect of pHF on CMA development may be a result of the large variation in pHFs characteristics. Different pHFs characteristics may be explained by whether the pHFs

are exclusively based on whey proteins or caseins or on whole milk. For example, lack of the preventive effect of partially hydrolysed whey on casein sensitisation development may be explained by the lack of casein derived peptides in this type of product and, as a consequence, lack of the oral tolerance induction towards caseins as explained in the study by Chikhi et al. [164]. In addition, different pHFs characteristics may be explained by the huge variation in DH, as illustrated by Graversen et al. [162].

Whereas the EAACI guideline from 2014 recommended the use of pHF for prevention of CMA [23], the recent EAACI guideline from 2021 has been updated and no longer provides specific recommendation for use of pHF [81] due to the lack of evidence for superior effect of pHF in preventing CMA. In line, the AAAAI [84] and ASCIA [83] guidelines likewise concluded that there is no evidence for recommending either against or for the specific use of pHF in CMA prevention. Guidelines in general emphasise the importance of considering each infant at high-risk of developing CMA independently, giving recommendations on infant formula based on their own individual circumstances [81,83,84].

Currently, there are no universal criteria for eHF production or batch-to-batch variance control, and different companies apply procedures with different enzyme hydrolysis conditions for their product manufacture, resulting in varying DH as well as varying peptides size distribution profiles [135,165]. Moreover, many producers do not have published data of their product safety and efficacy, including peptide profile and their residual immunogenicity and allergenicity [8,166]. Therefore, there is a need for uniform pre-clinical in vivo and in vitro testing procedures for evaluating residual allergenicity of future hypoallergenic infant formulas [153]. Variations in eHF characteristics result in different outcomes of their evaluation as a CMA management option both in human as well as animal studies.

eHFs are well tolerated by most cow's milk allergic infants. This is supported by animal studies showing that eHFs are suitable for CMA management, as they, in general, lack inherent allergenicity and do not induce clinical symptoms in cow's milk allergic animals [153,167]. Yet, several human studies have shown reactivity towards eHFs in some cow's milk allergic infants due to residual allergenicity still present even after extensive hydrolysis [8,166,168,169], indicating that eHF cannot be used for CMA management in all cow's milk-allergic infants.

Although eHFs are the best suited infant formulas for use in infants suffering from CMA who are not fully breastfed [104], they may, in addition to not being tolerated [144], be refused by some infants, as they may have a bitter taste due to hydrophobic amino acids that are exposed after hydrolysis [166,170,171], or be regarded as too expensive [1].

4.1.2. Fermentation

The ESPGHAN defines fermented formulas as infant and follow-up formulas that have been fermented with lactic acid producing bacteria during the production process but do not contain significant amounts of viable bacteria in the final product due to inactivation of the fermenting bacteria by for example heat treatment [104]. Hence, they are different from prebiotic or probiotic products in that they lack viable bacteria or prebiotic oligosaccharides but contain fermentation products, which might modulate gut immunity or gut microbiota, and promote allergy prevention [172]. Proteolytic enzymes secreted by lactic acid bacteria break down milk proteins, as displayed on Figure 3, leading to the degradation of IgE epitopes [137]. Indeed, peptides from the proteolysis of β-lactoglobulin and α-lactalbumin have been detected after fermentation of whey proteins by *Lactobacillus* species [173]. Destruction of β-lactoglobulin and casein epitopes could explain the reduction in binding of IgE from cow's milk allergic children to these proteins, as observed in several studies [174–176]. Infant formulas fermented by other bacteria than *Lactobacillus* species (e.g., *Bifidobacterium*) have also been investigated and have shown a capacity to strengthen the intestinal barrier in mice [177]. Moreover, a systematic review on the health benefits of fermented infant formulas concluded that there was evidence of reduced incidences of respiratory (e.g., bronchitis, wheezing) and gastrointestinal (e.g., vomiting, diarrhoea, colitis) allergic reactions in cow's milk allergic infants [178,179]. However, there

is not yet enough supporting evidence for the use of fermented formula for prevention or management of CMA [178,179], and more information on the exact composition and molecular structure of the fermented products as well as in-depth knowledge of mechanism of fermentation are needed for the optimisation of fermented infant formulas. Currently, no fermented infant formulas are commercially available.

4.1.3. Heat Treatment

Heat treatment of infant formulas or infant formula ingredients is used during the processing of these products to ensure microbiological safety and to obtain a long shelf life but not specifically to reduce milk allergenicity [133,134]. Pasteurisation (82 °C for 15 s or 94 °C for 30 s), in-can sterilisation (>110 °C for 10−30 min), spray drying (150–200 °C), or ultra-high temperature (UHT) treatment (135−150 °C for 2−6 s) are the most common heat treatments applied, and in some cases, they are combined [180,181]. However, information on the exact heating conditions is usually not available, as this information is commercially sensitive. Heating may induce modifications of amino acids in proteins, leading to changes in the protein structure and promoting interactions between proteins as well as between proteins and other ingredients in the infant formula (Figure 3) [182,183]. These modifications may affect, for instance, protein bioavailability, digestibility, as well as the presence and/or accessibility of IgE-binding epitopes and hence protein allergenicity [184–186]. The extent of the heat-induced alterations will be determined by the differences in and combination of processing, dependent on time, temperature, and rate of heating, as well as the composition of the infant formula.

Caseins lack well-defined secondary or tertiary structures, which render them very stable to high temperatures. Yet, heat treatment can lead to their precipitation and aggregation [187–190]. Whey proteins are in contrast generally susceptible to heat treatment and might undergo irreversible denaturation and aggregation as well as interact with casein micelles resulting in decreased solubility of the proteins [60,191–193]. Whereas β-lactoglobulin, the most abundant protein in whey, unfolds and aggregates at temperatures > 65 °C, α-lactalbumin is a bit more heat resistant, unfolding at temperatures > 70 °C, however, without formation of aggregates [184,190,194–197].

It has been reported that infant formulas are less heat stable than regular cow's milk, and whereas changes in protein secondary structure in the infant formula have been shown to begin at 50 °C, substantial changes in regular cow's milk were observed at 70 °C [198]. Changing the protein composition of infant formulas has significant effects on the formula heat stability. For example, in a study by Crowley et al., it was shown that increasing the ratio between α-lactalbumin and β-lactoglobulin increased the heat stability of the infant formula [199], which is in agreement with a study by Halabi et al., who observed that infant formulas with high α-lactalbumin and lactoferrin content were protected against heat denaturation of native whey proteins [200].

Generally, heat-induced denaturation caused by intense thermal processing promotes digestion of milk proteins [186,201,202]. For instance, digestion of caseins in infant formulas heated at 80 °C was faster than the digestion of the unheated counterpart, which could be explained by their smaller micelles covered by denatured whey protein aggregates, thus increasing the accessibility to proteases [203]. Further, another study showed that upon more intense temperatures of 120 °C, caseins were even more rapidly digested than after pasteurisation at 82 °C [204]. Likewise, several studies have shown that heating at temperatures between 75 and 90 °C denatures β-lactoglobulin and increases its accessibility to proteases and thus its digestibility [139,186,205,206].

Exposure to high temperatures during processing of infant formulas or infant formula ingredients can result in protein oxidation, where sulphur-containing amino acids as wells as aromatic amino acids are particularly susceptible to oxidation [207–212]. These modifications result in aggregation via covalent cross-linking or hydrophobic interactions as well as alteration of amino acids and protein conformation [213,214]. The extent of protein modifications seems to depend on the heating conditions. Moreover, oxidation-

based modifications, such as formation of dityrosine, increase surface hydrophobicity and viscosity and might be responsible for the reduced digestion of infant formulas [215,216]. Oxidation seems to be higher in infant formula compared to regular cow's milk [217].

In addition to oxidation, heat-induced glycation reactions between proteins and sugars in infant formula or infant formula ingredients might occur [181,218–221]. More specifically, interactions between the amino groups in proteins and reducing sugars, such as glucose or lactose in the milk, result in Maillard reaction products called Amadori products, which can undergo further reactions resulting in advanced glycation end products, such as carboxymethyl-lysine [181]. The glycation of lysine residues protects amino acids from proteolysis, decreasing protease accessibility, thus impairing protein digestion [182,186,222,223]. There is not uniformity in the degree of heat-induced modifications among similar infant formula ingredients from different manufacturers [224]. Generally, the degree of modifications and the number of modified proteins increase with higher temperatures and/or longer heating durations [221,225–227], and thermal processing during infant formula production has been shown to increase the presence of Maillard products compared to regular cow's milk [181,218]. Maillard products are not only present in the final infant formulas but already in the infant formula protein ingredients, as pasteurisation, emulsification, evaporation, spray drying, and sterilisation (Figure 2) of both whey and casein fractions may give rise to Amadori products [228,229].

The effect of heat treatment on allergenicity of cow's milk has been extensive studied, but only few reports have been described using infant formulas or infant formula ingredients [64,134,230,231]. In general, heat processing may have an impact on infant formula allergenicity either as a direct effect conferred by the protein modifications induced or as an indirect consequence of altered bioavailability and digestibility. Consequently, using optimised heat treatment as a processing method, infant formulas with low allergenicity could be produced. The molecular basis of modifying allergenicity is the destruction/masking of the IgE epitopes and/or exposure/formation of new epitopes by denaturation, aggregation, and amino acid modifications, thus reducing or enhancing IgE recognition [64]. Accessibility to β-lactoglobulin epitopes is temperature dependent. Temperatures < 90 °C increase β-lactoglobulin antigenicity due to protein unfolding and exposition of epitopes buried inside the native molecule; however, heating > 90 °C induces aggregation and amino acid modification, masking or destroying conformational and linear epitopes and thus decreasing both its antigenicity and allergenicity [231–235]. Contrary to whey proteins, caseins are thermostable and thus retain allergenicity even after extensive heat treatment [235].

The degree, length, and rate of heating; the type and concentration of reducing sugars; and the extent of glycation could be adjusted in order to influence allergenicity of infant formulas. In one study, conjugation of whey protein with maltose was shown to be an effective way to reduce the antigenicity of α-lactalbumin and β-lactoglobulin [230]. Another study showed that moderate glycation did only have a small effect on binding of IgE from cow's milk allergic patients to β-lactoglobulin, whereas a high degree of glycation masked IgE epitopes, reducing the recognition by IgE from allergic individuals [236].

Heat treatment affects allergenicity not only by modifying epitope recognition but also by hindering protein uptake and changing the uptake route. Indeed, heat-induced aggregation of whey proteins during pasteurisation impaired protein uptake through epithelial cells in a mouse model and thus protected against an allergic response [237]. In another study, Graversen et al. showed that partial heat-induced protein denaturation and aggregation of whey proteins changed the proteins route of uptake, being more efficiently transported through Peyer's patches, which might explain the reduced allergenicity of the modified whey proteins [238].

In general, studies considering the effect of heating on allergenicity indicated that the effect is very complex and dependent on many factors and not only on the heat stability and concentration of the proteins as well as the heat treatment regime but also on the presence of other components in the formula [134]. Yet, further research is needed to gather

knowledge on how to alter the processing parameters applied to infant formulas and the specific formulation to produce safe products for cow's milk allergenic individuals.

4.1.4. High Pressure

Non-thermal processing has been investigated as a method for reducing allergenicity of cow's milk proteins either as an alternative method to or in combination with thermal processing although only few studies have been performed with infant formula or infant formula ingredients [64,134]. One method investigated is HP treatment (200–600 MPa) although HP-based infant formulas are not commercially available yet. The HP process can affect non-covalent interactions, such as hydrophobic or electrostatic interactions between milk proteins, as well as affect the protein structure (Figure 3). Thus, HP-derived modifications may alter protein allergenicity. In fact, HP treatment (400 and 600 MPa) of whey proteins was shown to disrupt protein interactions and alter protein structure with resulting exposure of linear epitopes that were hidden in the native structure of the proteins [239]. Consequently, HP increases the allergenicity of whey proteins, depending on the exact time and degree of pressure, by increasing epitope accessibility and hence enhancing their allergenicity. Combination of HP and heat treatment (600 MPa, 40 °C) was shown to have a synergistic effect, which further increased the allergenicity of β-lactoglobulin [239,240]. On the contrary, studies performed by Chicón et al. showed that HP treatment (200 and 400 MPa) of whey proteins did not affect β-lactoglobulin allergenicity by means of binding to IgE [241].

A novel HP-based method that combines HP and short-term heat treatment, followed by an instant pressure drop to vacuum, has been investigated for reducing allergenicity of whey proteins and caseins. The protein conformational changes and aggregations observed resulted in opposite effects on the allergenicity for the whey and casein fractions, with a decreased allergenicity for whey proteins and an increased allergenicity for caseins [242].

4.1.5. Radiation

Microwave, ionisation (e.g., X-ray, high-energy electron beams, or γ-rays), ultraviolet (UV), or infrared radiation have gained much attention in the last decade because they induce conformational changes and denaturation of milk proteins (Figure 3), leading to the alteration in their epitopes [134,243–245]. For example, it has been reported that the allergenicity of β-lactoglobulin decreased by γ-radiation [246] and that the allergenicity of α-caseins and whey proteins decreased by UV treatment [244]. Yet, the effect on infant formulas has not been assessed.

4.1.6. Other Processing Technologies

Based on the literature available, it seems that other techniques, such as ultrasound and non-thermal atmospheric plasma, have no effect in reducing the allergenicity of whey proteins and caseins [247,248] although changes in the secondary structure of β-lactoglobulin were observed upon ultrasound application [247]. The impact of the processing techniques, pulse electric field, and ohmic heating on milk allergenicity has not been investigated yet but could be considered in the future, as it has been shown that a pulsed electric field induces structural modification of whey proteins [249].

5. Amino Acid-Based Infant Formulas

AAFs are exclusively based on free amino acids and are free from peptides derived from cow's milk proteins. They are used in infants with severe CMA where eHF cannot resolve all symptoms or in those cases where anaphylaxis occurs [250]. However, in some regions of the world, there is an excessive use of AAF due to the lack of proper diagnostic tools as well as resources to perform subsequent oral food challenge (OFC) for evaluation of acquisition of tolerance to cow's milk [251]. However, in EU, the use of AAF is only recommended if eHF cannot be used for CMA management.

Hypoallergenicity and thus the safety of AAF has been proven by several clinical trials showing that AAFs are well tolerated by infants suffering from severe CMA [252,253]. Nutritional aspects of AAF have also been assessed in order to evaluate whether infants fed with AAF have a normal growth rate when comparing with those fed with other types of infant formulas, which concluded that AAF supports a normal growth of infants [254–256].

6. Infant Formulas Based on Mammalian Milk Proteins

As previously stated in this review, cow's milk is the main source of proteins in dairy product manufacturing, including production of infant formulas, due to its great availability [129]. However, there is an increasing interest in the utility of other mammalian milk as a source of proteins in infant formula manufacture. At present, in EU, only milk proteins from cows and goats are allowed to be used in infant formula production in accordance to the EU legislation [7,257]. For dairy product manufacture, regular milk from non-cattle species, such as *Capra hircus* (goat), *Ovis aries* (sheep), and *Camelus dromedaries* (camel), contributes with ~17% of the global milk production. Milk from *Equus asinus* (donkey) or *Equus ferus caballus* (horse) are also gaining an increased interest for dairy product manufacture though on a smaller scale compared to goat, sheep, and camel milk [129].

Due to the population growth and hence the increasing need for protein sources, there is a demand for more and new dairy products, including those based on non-cattle milk [258]. Cow's milk is the most common source of proteins in infant formula both in the production of conventional and in the production of hydrolysed infant formulas [53]. The composition of mammalian milk differs between different animals and are different from breastmilk, with differences in total protein content, casein-to-whey protein ratio, protein composition, as well as differences in individual protein amino acid sequences.

Figure 4 displays the relationship between present and potential future mammalian milk sources for infant formula production discussed in this part of the review. Cow, goat, sheep, and camel belong to the order Artiodactyla; cow, goat, and sheep belong to the Ruminantia suborder and Bovidae family, while camel belongs to Tylopoda suborder and Camelidae family [259,260]. In addition, cow belongs to Bovinae subfamily, while goat and sheep belong to Caprinae subfamily. Further, goat belongs to Capra genius, and sheep belongs to Ovis genus [261]. Donkey and horse belong to another order called Perissodactyla and further belong to the same suborder (Hippomorphia), family (Equidae), subfamily (Equinae), and genus (Equus) and differ only in their species [262]. Artiodactyla and Perissodactyla orders are equally distanced from the Primates order (human).

Cross-reactivity between cow's milk proteins and counterpart proteins from other mammalian milk is an important factor when evaluating the usability of non-cattle milk in CMA prevention and management. Therefore, in Table 2, the amino acid sequence identity of allergens from cow's milk and their counterpart proteins in goat, sheep, camel, donkey, horse, and human milk is presented. Overall, from Table 2, it can be seen that goat and sheep milk proteins have a higher sequence identity with cow's milk allergens than proteins from camel, donkey, horse, and human milk. Furthermore, donkey and horse proteins have in general a lower sequence identity with cow's milk allergens than proteins from camel milk.

For CMA prevention, a certain degree of cross-reactivity between cow's milk and other non-cattle milk proteins is needed. Hence, in all probability, goat and sheep milk proteins would be a better choice for CMA prevention than camel, donkey, and horse milk proteins due to the high amino acid sequence identity with cow's milk proteins (Table 2). However, no studies have yet analysed the usability of non-cattle milk on CMA prevention. Thus, in the following sections, we will discuss the suitability of milk proteins from goat, sheep, camel, donkey, and horse milk as a protein source in alternative infant formulas for CMA management.

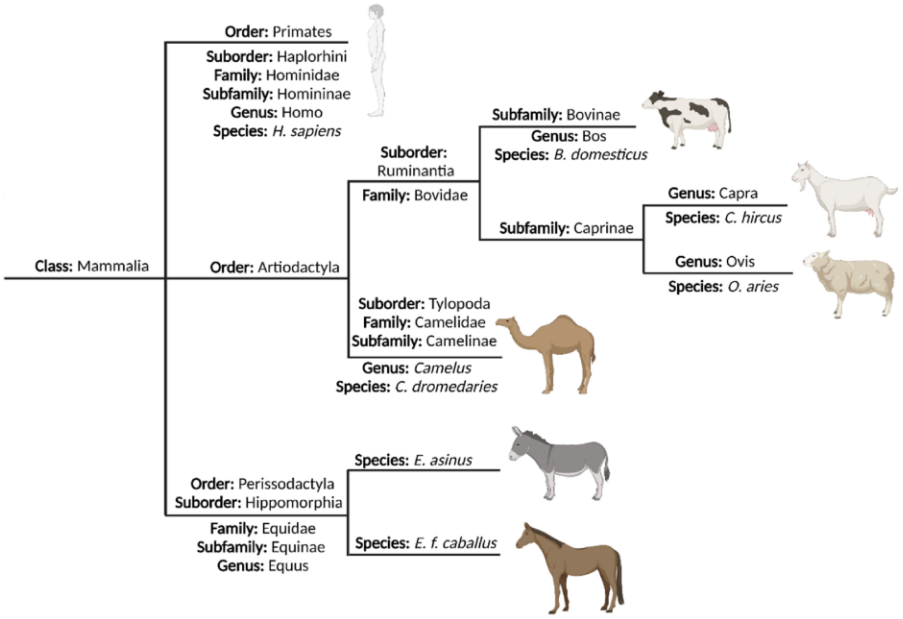

Figure 4. Relationship between present and potential future mammalian milk sources for infant formula manufacture. *B. Domesticus* (cow), *C. hirsus* (goat), *O. aries* (sheep), and *C. dromedaries* (camel) belong to the order Artiodactyla. Camel belongs to the suborder (Tylopoda), while cow, goat, and sheep belong to the same suborder (Ruminantia) as well as family (Bovidae), with cow belonging to Bovinae subfamily and goat and sheep belonging to Caprinae subfamily. Goat and sheep differ in their genius, where goat belongs to Capra, and sheep belongs to Ovis. Camels belong to Camelidae family, Camelinae subfamily, and genus *Camelus*. *E. asinus* (donkey), and *E.f. caballus* (horse) both belong to the order Perissodactyla as well as the same suborder (Hippomorphia), family (Equidae), subfamily (Equinae), and genus (Equus) and differ only in their species. Graphics created with BioRender.com.

Table 2. Amino acid sequence identity percentage (%) between cow and goat, sheep, camel, donkey, horse, and human milk proteins. Table modified from [263].

Protein	Goat	Sheep	Camel	Donkey [1]	Horse [1]	Human
α_{s1}-casein	91	91	67	57	55	33
α_{s2}-casein	88	88	47	46	46	NA
β-casein	88	89	56	60	57	55
k-casein	85	85	58	57	56	52
α-lactalbumin	95	95	60	47	60	74
β-lactoglobulin	93	93	NA	52	51	NA
Serum albumin	88	91	81	74	74	76
Lactoferrin	92	92	75	73	73	70

[1] Donkey and horse milk proteins were not included in amino acid sequence identity % table from [263]. Therefore, sequence alignments between cow's milk proteins and donkey and horse milk proteins were performed using CLS Main Workbench 8.0 and Uniprot and NCBI database. NA, not available. Accession number: β-casein: Cow: AAA30431; Donkey: XP_044622644; Horse: NP_001075321. α_{S1}-casein: Cow: AAA30429; Donkey: XP_014708642; Horse: AAK83668. α_{S2}-casein: Cow: NP_776953; Donkey: XP_044622647; Horse: NP_001164238. k-casein: Cow: CAA33034; Donkey: XP_014702750; Horse: AAK83669. α-lactalbumin: Cow: CAA29664; Donkey: XP_014705618; Horse: P08896. β-lactoglobulin: Cow: CAA32835; Donkey: P13613; Horse: AAC95385. Serum albumin: Cow: CAA41735; Donkey: AAV28861; Horse: P358747. Lactoferrin: Cow: AAA30610; Donkey: XP_044610851; Horse: NP_001157446.

6.1. Goat Milk

Milk from *Capra hircus* (goat) is widely available and used especially in the Mediterranean area of Europe as well as in some Western Europe countries, Asia, Australia, and New Zealand. Goat belongs to the Bovidae family, along with cow and sheep, and together with sheep, it belongs to the Caprinae subfamily (Figure 4). Goat milk is used as raw or pasteurised regular milk, in cheese, and in yoghurt production [264] as well as in the production of infant formulas as a source of proteins and micro and macro nutrients [265]. It contains a comparable amount of total protein to cow's milk, with a slightly higher ratio of caseins to whey proteins, being 84:16 compared to 80:20 for cow's milk [119]. Moreover, the profile of individual proteins differs, where it has been shown that goat milk contains significantly lower amount of α_{s1}-casein but significantly higher amount of α_{s2}-, β-, and k-casein compared to cow's milk [265,266]. On the other hand, the amount of specific whey proteins was found to be comparable for cow's and goat's milk [265]. Cow's and goat's milk proteins possess high amino acid sequence identities, as shown in Table 2. The amino acid sequence identities range between 85 and 95%, being slightly higher for whey proteins compared to caseins.

In 2012, European Food Safety Authority (EFSA) concluded that goat milk is a suitable source of proteins for infant formula [257]. Initially, goat milk was suggested as an alternative to hypoallergenic infant formulas for cow's milk allergic patients [267–269], but in recent years, there has been growing evidence supporting that infant formula based on intact goat milk proteins is not suitable as an alternative to hypoallergenic infant formulas for the management of CMA. In fact, DRACMA guideline [106] as well as an opinion by EFSA Scientific Panel [257] highlighted the importance of avoiding goat milk for CMA management.

Several studies have shown that IgE-mediated cow's milk allergic patients manifest cross-reactivity towards goat milk proteins. Based on in vivo and ex vivo analyses, they concluded that only few patients with CMA can tolerate goat milk and that most react to goat milk [270–272]. Conversely, there are some single cases reporting a tolerance to cow's milk in patients allergic to goat milk [273–276], indicating development of IgE specific for epitopes only present in goat milk proteins but absent in cow's milk proteins. For example, a study by Bernard et al. found an absence of cross-reactivity in patients allergic to goat milk with tolerance to cow's milk using β-casein [277]. The study concluded that the specificity of the IgE response to goat milk β-casein with concomitant lack of response to cow's milk β-casein was a result of difference in only three amino acids in the domain between amino acid 49 and 79, indicating that even small differences may indeed have a great impact on the IgE-binding capacity [277]. At present, no goat milk proteins are registered as allergens in the AllergenNomenclature [56] although studies have been reporting cases of goat milk allergy [273–276]. Allergenicity of goat milk has also been evaluated in animal models, where goat milk was shown to inhere a lower allergenicity than cow's milk [278,279].

Based on the current evidence, goat milk infant formula should be avoided in cow's milk allergic patients and should not be recommended for CMA management.

6.2. Sheep Milk

Milk from *Ovis aries* (sheep) is mainly available in countries such as China, New Zealand, Turkey, Greece, Syria, and Romania [280,281]. Together with cow and goat, sheep belongs to the Bovidae family, and together with goat, it belongs to the Caprinae subfamily (Figure 4). Sheep milk contains a higher amount of total protein compared to cow's milk, with a ratio of caseins to whey proteins comparable to that of cow's milk, i.e., 80:20 [281]. It contains a different profile of the specific proteins, with a higher amount of β- and α_{s2}-casein and lower amount of k- and α_{s1}-casein than cow's milk [281]. Similar to goat milk, sheep milk contains high amino acid sequence identities with counterpart cow's milk proteins, ranging between 85 and 95% and being slightly higher for whey proteins than caseins (Table 2).

Currently, sheep milk-based infant formulas are not approved in EU and hence are not commercially available but are available in China and New Zealand [282,283]. From the perspective of CMA management, currently, there is lack of studies evaluation usability of sheep milk. Yet, based on the large degree of homology between sheep and cow's milk proteins, similar to the homology between goat and cow's milk proteins, it must be anticipated that most cow's milk allergic infants may react to sheep milk-based infant formulas. However, several cases have been reported with allergic reactions toward sheep milk proteins after sheep cheese consumption in individuals who could tolerate and had no allergic reactions toward cow's milk [284–288]. Currently, no sheep milk proteins are registered as allergens in the AllergenNomenclature [56]

6.3. Camel Milk

Milk from *Camelus dromedaries* (camel) is an important source of nutrition in arid and semi-arid regions because camels can produce much more milk while on poor feed and lack of water than any other species [289,290]. In these regions, camel milk is used as raw or pasteurised regular milk or is used in dairy product manufacture for yoghurt, soft cheese, or ice creams. Together with cow, goat, and sheep, camel belongs to the Artiodactyla order but to a different family, namely the Camelidae family (Figure 4) [290]. In general, camel milk contains comparable amount of total proteins to cow's milk [291]. However, the ratio between caseins and whey proteins is different from that in cow's milk, with 74:16 in contrast to the 80:20 for cow's milk [129]. In addition, camel and cow's milk differ in their specific protein profile. First of all, β-lactoglobulin (Bos d 5), a protein found in the cow's milk whey fraction, also known as one of the major allergens [292], is not present in camel milk [291,293,294]. Moreover, camel milk contains lower amount of α_{s1}- and k-casein and higher amount of β-casein compared to cow's milk. In addition, the amount of α-lactalbumin and serum albumin is higher in the whey fraction of camel milk compared to the whey fraction of cow's milk [291]. The amino acid sequence identities between camel and cow's milk proteins range between 47 and 81%, being higher for whey proteins than caseins (Table 2).

Camel milk has gained an increasing interest in the last decade as a potential suitability source for infant formula manufacture, including manufacture of infant formulas for CMA management [290,295,296]. This is mainly due to its different profile of proteins and relatively low amino acid sequence identities with cow's milk proteins especially in comparison to goat and sheep milk proteins [263]. At present, no infant formula based on camel milk is available on the EU market; however, in the Middle East, a stage three toddler formula based on camel milk is commercially available.

At present, there is a number of clinical trials evaluating the usefulness of camel milk as an alternative milk for patients allergic to cow's milk, and the results are consistent. A study by Navarrete-Rodríguez et al. showed no clinical symptom manifestation after two weeks of consumption of camel milk in patients with confirmed CMA [297]. Moreover, several studies using in vivo method, such as SPT, showed a low level of reactivity towards camel milk, with <20% of the cow's milk allergic patients reacting [298–300].

In addition, there are a number of studies performing ex vivo analyses using blood from cow's milk allergic patients for antibody reactivity evaluation, concluding reduced or no reactivity of specific IgE towards camel milk proteins [301–304].

Currently, there is one case that reported anaphylaxis after camel milk consumption in an atopic child who had never experienced allergy to cow's milk proteins [305]. At present, no proteins from camel milk are registered as allergens in the AllergenNomenclature [56].

An animal study evaluating cross-reactivity between cow's and camel milk proteins showed that there was low cross-reactivity between camel and cow's milk proteins, with lower cross-reactivity between caseins than between whey proteins [263]. The study also showed that the linear epitopes were predominant in casein cross-reactivity, while conformational epitopes prevailed in whey protein cross-reactivity.

Camel milk may have a potential to be used as a source of proteins in infant formulas for CMA management. However, further investigations are required.

6.4. Donkey Milk

Milk from *Equus asinus* (donkey) is mostly common in the Mediterranean countries, such as Spain, Greece, France and Italy, as well as Asian and African countries [306]. Together with the horse, donkey belongs to another order than cow, goat, sheep, and camel, namely the Perissodactyla order (Figure 4).

Donkey milk contains around two times less proteins in comparison to cow's milk [307]. In addition, it has a very different casein-to-whey protein ratio, with 58:42 in contrast to 80:20 for cow's milk [119,308]. Donkey milk contains a lower amount of α_{s2}-casein but significantly higher amount of α_{s1}-, k-, and β-casein than cow's milk [308]. In general, the protein sequence identities between donkey and cow's milk proteins are low when compared with proteins from goat or sheep milk. The amino acid sequence identities range between 47 and 74% for whey proteins and between 46 and 60% for caseins (Table 2).

As a non-cattle milk, donkey milk is gaining increasing interest, especially in Italy [307], for its potential usability in infants with CMA and thus for the future application as a protein source in infant formula manufacture. Several clinical studies have been performed to evaluate the safety of donkey milk in cow's milk allergic patients using in vivo or ex vivo methods. The outcome of the studies were consistent, where tolerance to donkey milk after OFC was reported in more than 80% of the cow's milk allergic patients enrolled in all studies [309–313].

Vita et al. compared the level of tolerance towards goat and donkey milk in patients with atopic dermatitis and CMA [314] and showed that donkey milk was tolerated by 88% of patients in comparison to none for goat milk and that consumption of donkey milk improved the atopic dermatitis.

At present, there are a number of cases reported in relation to donkey milk protein allergy without concomitant CMA, including two patients who developed symptoms after donkey milk consumption [315,316] and one manifesting clinical symptoms after inhalation, showing respiratory allergy [317]. In addition, a case of skin contact allergy was reported where a patient developed urticaria after using donkey milk containing cosmetics [315]. Based on two cases reported by Martini et al. [308,315], lysozyme was identified as an allergen in donkey milk and included in the AllergenNomenclature [56].

Based on the current evidence showing a high level of tolerance to donkey milk in patients with CMA, donkey milk may be a potential source of proteins in future infant formulas for CMA management. However, further investigations are needed.

6.5. Horse Milk

Milk from *Equus ferus caballus* (horse) is mainly popular in countries such as Mongolia, Kazakstan, Kyrgyzstan, and Tajikistan [318,319]. Together with donkey, horse belongs to another order than cow, goat, sheep, and camel, namely the Perissodactyla order (Figure 4).

Horse milk contains two times less protein than cow's milk and has a comparable casein-to-whey protein ratio to that of donkey milk, i.e., 56:44, which is very different from that of cow's milk at 80:20 [119]. Horse milk has a lower α_{s1}- and α_{s2}-casein content and a higher α-lactalbumin content than cow's milk [318]. The amino acid sequence identities with cow's milk proteins range between 51 and 74% for whey proteins and between 46 and 57% for caseins (Table 2). Like donkey milk, horse milk is gaining increased interest for its potential usability for cow's milk allergic infants and children.

A study by Businco et al. showed that horse milk was tolerated by 96% children with CMA by means of an OFC [320], and in a study by Fotschki et al. using an animal model, it was shown that horse milk consumption decreased total IgE level in mice sensitised to cow's milk [321]. In another animal model, the allergenicity of horse milk was shown to be lower than the allergenicity of cow's and goat milk [322].

Cases of horse milk allergy without concomitant CMA have been reported. One case report described skin contact allergy after application of a body cream containing horse milk as an ingredient with manifestation of swelling and itchiness but also horse milk α-lactalbumin-positive IgE in serum [323]. Moreover, two cases of horse milk allergy have been reported after its consumption, without concomitant CMA [324,325].

Horse milk lysozyme is registered as an allergen in the AllergenNomenclature [56] due to a 99% sequence identity with donkey milk lysozyme [315]. In addition, horse serum albumin has been registered as an airway allergen [56,326]. Yet, as serum albumin is a protein also found in milk, patients with confirmed horse serum albumin inhalation allergy should also avoid horse milk consumption.

Current evidence indicates that horse milk, just as donkey milk, possesses a high level of tolerance in patients with CMA. However, more studies are required for a further evaluation of its usefulness as a protein source in infant formulas for CMA management.

7. Plant-Based Infant Formulas

Infant formulas, as substitutes to breastmilk, are largely based on dairy proteins. Yet, in recent years, there has been a great focus on alternative protein sources of plant origin—not only as a substitute to cow's milk-based formulas for infants suffering from CMA or cow's milk intolerance but also for taste preference, vegan habits, environmental, climate, and ethical reasons [9,10]. Indeed, there is an immense focus on providing more sustainable and climate-friendly dietary solutions for the future [327–329].

In general, the demand for plant-based beverages has increased throughout the world in the last years [330–332] and can be divided into five categories: cereal-based (oat, rice, corn, spelt), legumes-based (soy, peanut, lupin, cowpea, chickpea), nut-based (almond, coconut, cashew, hazelnut, Brazil nut, pistachio), seed-based (sunflower, sesame, hemp), and pseudocereal-based (quinoa, teff, amaranth) beverages [10,144,333–335]. It has been reported that parents and caretakers are increasingly feeding infants and young children with such plant-based beverages as alternatives to cow's milk-based products, including as substitutes for cow's milk-based infant formulas [336,337]. The quality of plant-based alternatives varies and may not necessarily address the nutritional requirements of infants and small children [144,331,333,338]. Thus, it appears that there is no health benefit of plant-based alternatives to cow's milk-based products in small children but rather a potential health risk related to frequent consumption of these plant-based alternatives if the child's diet is not properly managed [331,338]. In fact, case-based evidence with severe malnutrition caused by plant-based beverage feeding in infants down to 1 months of age has been reported [337,338] where, in some cases, the infants were fed with the plant-based beverages already from birth [338].

Indeed, there has been recommendation against plant-based beverages for small children [338], and for infants up to an age of 12 months, it is recommended only to use appropriate commercial infant formulas as alternatives to breastmilk [336]. Only a few commercially available infant formulas based on plant proteins exist, and these are manufactured from either soy or rice proteins (Figure 5). In the EU, the only source of protein allowed in infant and follow-up formulas are cow's milk, goat milk, soy, as well as hydrolysed proteins [7].

For infants with severe CMA that cannot tolerate eHF, alternatives to AAF are soy- and hydrolysed rice-based infant formulas. These infant formulas are, in general, well tolerated and considered a second choice for cow's milk allergic infants and small children in some countries [1]. Yet, ESPGHAN and EAACI recommend against the use of soy protein-based formulas in infants below the age of 6 months [23,104,339]. Similar to eHF, plant-based infant formulas for management of CMA in infants should also be tolerated by at least 90% of the children with CMA, with a confidence interval of 95% [6,80]. However, in some cases, plant-protein based formulas may not prove hypoallergenic for cow's milk allergic infants [336].

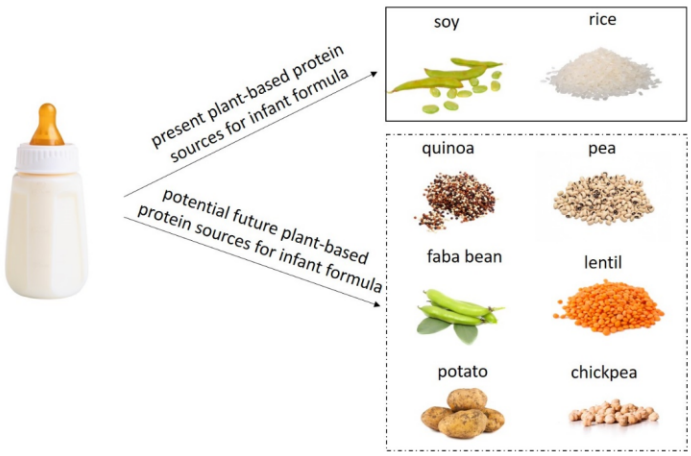

Figure 5. Present and potential future plant-based protein sources for infant formula manufacture. Present plant-based protein sources for commercially available infant formula manufacture are soy and rice. Potential future plant-based protein sources suggested for infant formula manufacture are quinoa, pea, faba bean, lentil, potato, and chickpea. Pictures were purchased from Colourbox.com.

7.1. Soy-Based Infant Formulas

Soybean is a legume crop originating from East Asia with a high-quality content of proteins comprising up to 40% of the dry weight [339,340]. Soy-based infant formulas are available in many countries throughout the world, with the largest market being in North America [341]. Soy-based infant formulas have been commercially available for more than a century although they have changed throughout this time [340,342,343]. At first, the soy-based infant formulas were altered from being based on soy flour to soy protein isolate in order to obtain a higher digestibility and a lower content of fibres and phytates [343,344]. Later, the soy-based infant formulas were fortified with the amino acids methionine, taurine, and carnitine as well as with choline and inositol [342,343]. Most recently, the soy-based infant formulas have been supplemented with LCPUFAs [344]. Despite the initiatives to improve soy-based infant formulas over time in order for them to be safe and to meet the nutritional need of infants comparable to that of cow's-milk-based infant formulas [118,342,343], concerns have been raised regarding potential risks due to the phytate and phytoestrogen content as well as nutritional deficiencies [1,339,342]. Yet, based on a meta-analysis, Vandenplas et al. concluded that soy-based infant formulas are a safe alternative to cow's milk-based infant formulas [343].

Soy allergy is less prevalent than CMA although it affects around 0.3–0.4% of small children [345,346], and according to AllergenNomenclature, eight allergens have been identified [56]. However, as soy-based infant formulas do not contain cow's milk proteins and lactose, it may be a choice for infants suffering from CMA or cow's milk intolerance, and for most cow's milk allergic infants, soy-based infant formulas are also well-tolerated [144]. Before the introduction of eHF on the market, it was the only formula available for the feeding of infants with CMA [339,347]. However, co-sensitisation to cow's milk and soy proteins is common, whereas cross-reactivity between cow's milk and soy proteins is not [346] even though it has been demonstrated [348]. It has been reported that up to 50% of cow's milk allergic infants may react to the soy-based infant formulas [339,347] although different studies based on OFC have shown lower yet varying results, revealing clinically relevant reactions to soy or soy-based infant formula in 3% [349], 7% [350], 10% [351], or 14% [352] of cow's milk allergic infants, respectively.

The use of soy-based infant formulas in the prevention of atopic diseases in high-risk infants seems controversial [339,347], with studies showing some prophylactic effect of soy-based infant formulas when compared to cow's milk-based formulas [353,354], whereas other studies did not show such effect [355,356]. Yet, in a meta-analysis, Osborn and Sinn concluded that soy-based infant formulas cannot be recommended for use in the prevention of food allergies in high-risk infants [357], which is generally supported by most guidelines [358]. For example, EAACI recommends against using soy-based formula in the first 6 months of life as a means of preventing food allergy [81]. Controversies also exist for the acquisition of tolerance to cow's milk when soy-based infant formulas are used in the management of CMA, where one study reported that soy-based infant formula was more effective than eHF in tolerance acquisition [359], whereas another study showed that an eHF was more effective than was a soy-based infant formula [360], and a third study showed no differences between the formula choice on tolerance acquisition [355].

Due to the perceptual nutritional disadvantages and allergenic potential of soy-based infant formulas, ESPHGAN, EAACI, and AAP do not recommend giving soy-based infant formulas to infants below the age of 6 months [23,104,118,339]. Yet, ESPGHAN and AAP state that soy-based infant formulas may be considered in infants above the age of 6 months when complementary feeding has been initiated and in the absence of soy allergy for infants suffering from CMA and when parents wish to exclude products of animal origin or believe that eHFs are too expensive [104,339].

7.2. Hydrolysed Rice-Based Infant Formulas

Rice is a cereal believed to originate from Asia and has a rather low content of proteins comprising around 8% of the dry weight [361–363]. Rice-based infant formulas have for two decades been available in Spain, Italy, and France, where they are categorised as "foods for special medical purposes" (FSMP) [144,364,365] according to European law [366,367]. These are foods intended for dietary management, under medical supervision, of patients who suffer from certain diseases, disorder, or medical condition [366]. A requirement for formulas based on rice is that these formulas shall be based on hydrolysed rice proteins to obtain higher water solubility and digestibility as well as be fortified with the amino acids lysine, threonine, and tryptophan [365]. While hydrolysed rice-based infant formulas are available in Spain, Italy, and France, they are still not available in other European countries as well as in the U.S., Canada, Australia, and New Zealand but are, on the other hand, emerging in a growing number of African, Asian, and South American countries [364].

Commercially available hydrolysed rice-based infant formulas are in general well-tolerated and support the normal growth of infants [365,368,369] and have been reported to be growing in popularity due to their proven safety and due to being a cheaper choice than eHF [144,364,365].

The prevalence of rice allergy in small children is common in countries where it is frequently eaten but is generally low in Western countries [144,365,370,371], and according to AllergenNomenclature, only two rice allergens have been identified being categorised as respiratory allergens [56]. This as well as the absence of cross-reactivity between rice and cow's milk proteins makes rice-based formulas well tolerated in children with CMA, and only a limited number of cases of allergic responses toward hydrolysed rice-based infant formulas has been implied [364]. In two studies, it has been reported that cow's milk allergic infants showed reactivity to the hydrolysed rice-based infant formulas with specific IgE > 0.35 kU/L or with positive SPT although no clinical reactivity was observed upon OFC [370,372], whereas in another study, no reactivity to the hydrolysed rice-based infant formula was revealed [373].

When it comes to acquisition of tolerance to cow's milk when hydrolysed rice-based infant formulas are used in the management of CMA, there are conflicting evidence, where one study showed no differences when compared to eHF [373], another study showed that hydrolysed rice-based infant formulas were more effective than eHF [359], and a third

study showed the eHF to be more effective than hydrolysed rice-based infant formula in acquisition of tolerance to cow's milk [360].

Hydrolysed rice-based infant formulas have, in general, proven to be a safe choice for cow's milk allergic infants, and the DRACMA guidelines suggest that hydrolysed rice-based infant formulas may be an equivalent to AAF as second choice for infant formula feeding if eHFs cannot be tolerated for the management of CMA [296]. Yet, increasing amounts of data are being published even supporting the use of hydrolysed rice-based formulas as a possible first choice for infants with CMA [107]. However, ESPGHAN, EAACI, and AAP do not mention hydrolysed rice-based infant formulas in their guidelines [23,80,104].

7.3. Potential Future Plant-Based Infant Formulas

In contrast to soy-based and hydrolysed rice-based infant formulas, where there is a great amount of literature available, there is not much literature on other plant-based alternatives to cow's milk based infant formulas. Soy- and hydrolysed rice-based formulas are presently the only infant formulas nutritionally adapted for infants that are commercially available [107]. Yet, in the last decade, there has been an increasing interest in investigating new and potential future plant-based infant formulas either as complete plant-based infant formulas or as partial plant-based infant formulas, where only a proportion of the cow's milk proteins are substituted with plant proteins.

Several plants have been suggested as potential suitable protein sources for new infant formulas, these being quinoa [374], pea [375–377], faba bean [375–377], lentil [378], potato [376,379], and chickpea [380,381], as shown on Figure 5. Nevertheless, before any of these plant-based protein sources can be used in infant formulas, they would need to comply with the Regulation EU 2016/127 [114], and for some, they may even be regarded as novel foods, as new processing procedures may be a necessity to provide protein isolates and hence require an EU authorisation as a novel food [382].

Most of the studies concerning new plant-based infant formulas have focused primarily on physicochemical and functional properties as well as digestibility, as integration of new protein sources in formulas for infant nutrition rely on some "standard" properties, such as solubility, emulsification, and stability, as well as nutritional quality required to meet the needs of infants [114,383].

For legumes, lentil proteins have been suggested as an alternative to cow's milk proteins for infant formulas due to a high protein content of around 20–30% and a good amino acid profile [378]. In a recent study, Alonso-Miravalles et al. [378] investigated the physicochemical properties of a lentil protein-based formulation in comparison to two conventional plant-based infant formulas: one based on soy protein and one based on rice proteins. They concluded that from a physicochemical and nutritional perspective, lentil proteins are a good alternative to other sources of plant proteins for infant formulas [378]. However, allergenicity to lentil seems to be well documented in some countries and may lead to severe allergic reactions [384,385], and according to the AllergenNomenclature, three lentil allergens have been identified [56]. Lentil allergy has been reported as one of the most common non-priority (emerging) food allergies, which could be envisioned to increase in prevalence due to its increasing popularity [386].

The legume faba bean is also known as fava bean or broad bean. In studies of partial substitution of cow's milk proteins with faba bean proteins in infant formulas [375–377], it was reported that physicochemical properties of the formula were affected to some degree by the protein substitution [377]. It was shown that the digestibility of the formula was higher by substituting 50% of the cow's milk proteins with faba bean protein in a dynamic in vitro model [375], whereas there were no significant differences observed in a static in vitro digestion model [376]. Overall, partial substitution of cow's milk proteins with faba bean proteins resulted in formula physicochemical and digestibility properties more closely resembling those of fully cow's milk-based infant formula than if cow's milk proteins were substituted with rice proteins [376]. Thus, Le Roux et al. concluded that faba bean proteins could be a good candidate for partial substitution of cow's milk proteins in infant

formulas [375,376] although process parameters would need to be optimised to meet infant formula quality criteria [377]. Allergy to faba beans has only been demonstrated in few studies; yet, these indicate that faba beans contain clinically relevant allergens [387,388] although no faba bean allergens are listed in the AllergenNomenclature [56].

Similarly, the legume pea has also been studied in a partial substitution of cow's milk proteins for infant formulas [375–377], with the main difference compared to faba beans being that substituting 50% of cow's milk proteins with pea proteins resulted in a lower digestibility [375]. Pea allergy is well documented [389], and according to the AllergenNomenclature, pea contains three identified allergens [56]. Like lentil allergy, pea allergy has been reported as one of the most common non-priority (emerging) food allergies [384,386], and with the increasing use of pea protein isolate in various foods, it must be anticipated that allergy to pea will increase [384,389].

Chickpea, which is also a legume, has been suggested as a potential future protein source in infant formulas [380,381]. In evaluating the nutritional value of chickpea-based formulations, it was concluded that chickpea-based formulas may be a potential future alternative to cow's milk-based formulas for infants above the age of 6 months [380,381]. Allergy to chickpea has been reported in few studies, with identification of two allergens [384,385], though only one chickpea allergen is listed in the AllergenNomenclature [56]. For pseudocereals, quinoa has been suggested as an alternative source to cow's milk proteins in follow-up formulas due to its high-quality protein content [374]. Quinoa seems to be increasingly appreciated as an excellent gluten-free protein source for a wide range of consumers, including infants [374,390,391]. Yet, concerns have been raised regarding the high amount of saponins in quinoa, which inhere adjuvant capacity and may affect intestinal permeability [392]. Only few studies have investigated allergy to quinoa proteins, showing that quinoa may contain allergenic proteins [393–396]. However, no quinoa allergens are listed in the AllergenNomenclature [56]. Nevertheless, it should be acknowledged that since quinoa has not been a standard part of the Western diet, larger amounts and frequent consumption of quinoa might enhance the development of allergies.

Potato is a root vegetable commonly ingested throughout the world. Although potato allergy is uncommon, cases of potato allergy have been reported [397], and according to the AllergenNomenclature, four potato allergens have been identified [56]. Recently, a patent application on an infant formula for cow's milk allergic infants wherein the major source of protein is potato proteins has been filed [WO2018050705] [379].

Currently, plant-based infant formulas based on other proteins than those derived from soy and hydrolysed rice are not allowed in infant formulas according to the Regulation EU No 609/2013 [7]; however, in the latest Regulation EU 2016/127, it is stated that in order to ensure innovation and product development, other ingredients not covered by the specific requirement of the Regulation should be possible, provided their suitability for infant feeding has been demonstrated and authorised [114]. Recently, some toddler formulations have been marketed based on pea, rice, buckwheat, and almond that meet the nutritional need of toddlers [107].

8. Conclusions

In this review, we provided an overview of current and potential future options for protein sources in infant formulas in the context of CMA prevention and management. Breastfeeding should always be a first choice of infants feeding both in general as well as in CMA prevention and management. Cow's milk-based infant formulas are the main substitute to mother's milk if breastfeeding is not possible, insufficient, or not chosen. In addition to cow's milk-based infant formulas, infant formulas based on goat milk, soy, or hydrolysed rice proteins are also available on the EU market.

There are presently no specific recommendations for use of any particular infant formula for CMA prevention, but it was, until recently, recommended to use pHF for the prevention of CMA in high-risk infants. For CMA management, eHFs are currently recommended as a first choice; however, if the eHF is not tolerated or if the infant suffers

from severe CMA, AAF or alternatively a hydrolysed rice-based formula may be a second choice. Yet, infant formulas based on modified cow's milk proteins, other mammalian milk proteins, and plant-based proteins have been investigated as potential future protein sources for infant formulas both in general and for cow's milk allergic infants.

Processing technologies in addition to hydrolysis, such as heat treatment, fermentation, HP, and irradiation, are methods known to modify proteins by means of breakdown, denaturation, aggregation, oxidation, and glycation and thus have the potential to reduce allergenicity.

Whereas goat- and sheep-milk-based infant formulas may be good alternatives to conventional cow's milk-based infant formulas, they are not a suitable choice for CMA management due to the high homology of their proteins to cow's milk allergens and hence the great risk of cross-reactivity. Camel, donkey, and horse milk may, however, provide a better alternative for CMA management due to the lower protein homology and hence lower cross-reactivity to counterpart cow's milk allergens. Camel milk, which lacks the protein β-lactoglobulin, would have the potential to be used for infants allergic to primarily cow's milk β-lactoglobulin. Yet, the amount of evidence for the suitability of alternatively mammalian milk for use in cow's milk allergic infants is still limited, and thus, more research is needed.

Infant formulas based on soy and hydrolysed rice proteins are plant based-infant formulas presently available on the market in some countries and recommended as a potential second choice for CMA management in some countries. However, ESPGHAN, EAACI, and AAP do not recommend the use of soy-based infant formulas in infants below 6 months of age. Infant formulas based on alternative plant proteins have, in the last decade, gained an increasing interest as a sustainability and vegan alternative to milk-based infant formulas, and several plants have been suggested as a protein source for future infant formulas. With the current focus on more sustainable and climate-friendly dietary solutions, we could foresee that much more research would be conducted for evaluation of the suitability of plant-based infant formulas not only for the CMA management but as a general choice for infant nutrition.

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References

1. Fiocchi, A.; Dahda, L.; Dupont, C.; Campoy, C.; Fierro, V.; Nieto, A. Cow's milk allergy: Towards an update of DRACMA guidelines. *World Allergy Organ. J.* **2016**, *9*, 35. [CrossRef] [PubMed]
2. Arenz, S.; Rückerl, R.; Koletzko, B.; Von Kries, R. Breast-feeding and childhood obesity—A systematic review. *Int. J. Obes.* **2004**, *28*, 1247–1256. [CrossRef] [PubMed]
3. Belfort, M.B. The Science of Breastfeeding and Brain Development. *Breastfeed. Med.* **2017**, *12*, 459–461. [CrossRef] [PubMed]
4. WHO. Infant and young child nutrition: Global strategy on infant and young child feeding. In Proceedings of the Fifty Fifth World Health Assembly, Geneva, Switzerland, 13–18 May 2002; Volume 53, pp. 1–18.
5. Martin, C.R.; Ling, P.R.; Blackburn, G.L. Review of infant feeding: Key features of breast milk and infant formula. *Nutrients* **2016**, *8*, 279. [CrossRef] [PubMed]

6. European Comission. Commission Directive 2006/141/EC of 22 December on infant formulae and follow-on formulae and amending Directive 1999/21/EC. *Off. J. Eur. Union* **2006**, *49*, 1–33.
7. European Comission. Commission Directive 2013/46/EU of 28 August 2013 amending Directive 2006/141/EC with regard to protein requirements for infant formulae and follow-on formulae. *Off. J. Eur. Union* **2013**, *46*, 16–19.
8. Nutten, S.; Maynard, F.; Järvi, A.; Rytz, A.; Simons, P.J.; Heine, R.G.; Kuslys, M. Peptide size profile and residual immunogenic milk protein or peptide content in extensively hydrolyzed infant formulas. *Allergy Eur. J. Allergy Clin. Immunol.* **2020**, *75*, 1446–1449. [CrossRef]
9. McCarthy, K.S.; Parker, M.; Ameerally, A.; Drake, S.L.; Drake, M.A. Drivers of choice for fluid milk versus plant-based alternatives: What are consumer perceptions of fluid milk? *J. Dairy Sci. Off. Publ. Am. Dairy Sci. Assoc.* **2017**, *100*, 6125–6138. [CrossRef]
10. Silva, A.R.A.; Silva, M.M.N.; Ribeiro, B.D. Health issues and technological aspects of plant-based alternative milk. *Food Res. Int.* **2020**, *131*, 108972. [CrossRef]
11. Dahdah, L.; Sato, S.; Hossny, E.; Irani, C.; Said, M.; Scadding, G. The Global Problem of food allergy—Information Sheet. World Allergy WeekWorld Allergy Week; pp. 1–8. 2019. Available online: https://www.worldallergy.org/UserFiles/file/WAO-2019-Food-Allergy-Information-Sheet.pdf (accessed on 15 January 2022).
12. Tang, M.L.K.; Mullins, R.J. Food allergy: Is prevalence increasing? *Intern. Med. J.* **2016**, *47*, 256–261. [CrossRef]
13. INFOSAN IFSAN. Information Note No. 3/2006—Food Allergies; pp. 9–12. 2006. Available online: https://www.who.int/foodsafety/fs_management/No_03_allergy_June06_en.pdf (accessed on 15 January 2022).
14. Teuber, S.; Beyer, K.; Comstock, S.; Wallowitz, M. The Big Eight Foods: Clinical and Epidemiological Overview. In *Food Allergy*; ASM Press: Washington, DC, USA, 2006; pp. 49–79.
15. Zepeda-Ortega, B.; Goh, A.; Xepapadaki, P.; Sprikkelman, A.; Nicolaou, N.; Hernandez, R.E.H.; Latiff, A.H.A.; Yat, M.T.; Diab, M.; Hussaini, B.A. Strategies and Future Opportunities for the Prevention, Diagnosis, and Management of Cow Milk Allergy. *Front. Immunol.* **2021**, *12*, 1877. [CrossRef] [PubMed]
16. Loh, W.; Tang, M.L.K. The epidemiology of food allergy in the global context. *Int. J. Environ. Res. Public Health* **2018**, *15*, 2043. [CrossRef] [PubMed]
17. Longo, G.; Berti, I.; Burks, A.W.; Krauss, B.; Barbi, E. IgE-mediated food allergy in children. *Lancet* **2013**, *382*, 1656–1664. [CrossRef]
18. Skripak, J.M.; Matsui, E.C.; Mudd, K.; Wood, R.A. The natural history of IgE-mediated cow's milk allergy. *J. Allergy Clin. Immunol.* **2007**, *120*, 1172–1177. [CrossRef] [PubMed]
19. Ponce, M.; Diesner, S.C.; Szépfalusi, Z.; Eiwegger, T. Markers of tolerance development to food allergens. *Allergy Eur. J. Allergy Clin. Immunol.* **2016**, *71*, 1393–1404. [CrossRef]
20. Berin, M.C. Mechanisms that define transient versus persistent food allergy. *J. Allergy Clin. Immunol.* **2019**, *143*, 453–457. [CrossRef]
21. Sampson, H.A.; O'Mahony, L.; Burks, A.W.; Plaut, M.; Lack, G.; Akdis, C.A. Mechanisms of food allergy. *J. Allergy Clin. Immunol.* **2018**, *141*, 11–19. [CrossRef]
22. Gupta, R.S.; Lau, C.H.; Sita, E.E.; Smith, B.; Greenhawt, M.J. Factors associated with reported food allergy tolerance among US children. *Ann. Allergy Asthma Immunol.* **2013**, *111*, 194–198.e4. [CrossRef]
23. Muraro, A.; Werfel, T.; Hoffmann-Sommergruber, K.; Roberts, G.; Beyer, K.; Bindslev-Jensen, C.; Cardona, V.; Dubois, A.; Dutoit, G.; Eigenmann, P.; et al. EAACI Food Allergy and Anaphylaxis Guidelines: Diagnosis and management of food allergy. *Allergy Eur. J. Allergy Clin. Immunol.* **2014**, *69*, 1008–1025. [CrossRef]
24. Anagnostou, K. Oral Immunotherapy for Food Allergy: What Have We Achieved So Far? *EMJ Allergy Immunol.* **2017**, *2*, 94–99.
25. Burbank, A.J.; Sood, P.; Vickery, B.P.; Wood, R.A. Oral Immunotherapy for Food Allergy. *Immunol. Allergy Clin. N. Am.* **2016**, *36*, 55–69. [CrossRef] [PubMed]
26. ADP101 for Oral Immunotherapy in Food-Allergic Children and Adults. Available online: https://clinicaltrials.gov/ct2/show/results/NCT04856865 (accessed on 15 January 2022).
27. Food and Drug Administration. PALFORZIA. Available online: https://www.fda.gov/vaccines-blood-biologics/allergenics/palforzia (accessed on 15 January 2022).
28. Vierk, K.A.; Koehler, K.M.; Fein, S.B.; Street, D.A. Prevalence of self-reported food allergy in American adults and use of food labels. *J. Allergy Clin. Immunol.* **2007**, *119*, 1504–1510. [CrossRef] [PubMed]
29. Sicherer, S.H.; Sampson, H.A. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *J. Allergy Clin. Immunol.* **2014**, *133*, 291–307.e5. [CrossRef]
30. Kazatsky, A.M.; Wood, R.A. Classification of Food Allergens and Cross-Reactivity. *Curr. Allergy Asthma Rep.* **2016**, *16*, 22. [CrossRef] [PubMed]
31. Wright, B.L.; Walkner, M.; Vickery, B.P.; Gupta, R.S. Clinical Management of Food Allergy. *Pediatr. Clin. N. Am.* **2015**, *62*, 1409–1424. [CrossRef]
32. Lieberman, J.A.; Sicherer, S.H. Quality of life in food allergy. *Curr. Opin. Allergy Clin. Immunol.* **2011**, *11*, 236–242. [CrossRef] [PubMed]
33. Lieberman, J.A.; Weiss, C.; Furlong, T.J.; Sicherer, M.; Sicherer, S.H. Bullying among pediatric patients with food allergy. *Ann. Allergy Asthma Immunol.* **2010**, *105*, 282–286. [CrossRef]
34. Rajan, J.; Laubach, S. Child and Parental Reports of Bullying in a Consecutive Sample of Children With Food Allergy. *Pediatrics* **2013**, *132*, 23–24. [CrossRef]
35. Yu, W.; Freeland, D.M.H.; Nadeau, K.C. Food allergy: Immune mechanisms, diagnosis and immunotherapy. *Nat. Rev. Immunol.* **2016**, *16*, 751–765. [CrossRef]

36. Czolk, R.; Klueber, J.; Sørensen, M.; Wilmes, P.; Codreanu-Morel, F.; Skov, P.S.; Hilger, C.; Bindslev-Jensen, C.; Ollert, M.; Kuehn, A. IgE-Mediated Peanut Allergy: Current and Novel Predictive Biomarkers for Clinical Phenotypes Using Multi-Omics Approaches. *Front. Immunol.* **2021**, *11*, 3653. [CrossRef]
37. Broekman, H.C.H.; Eiwegger, T.; Upton, J.; Bøgh, K.L. IgE—The main player of food allergy. *Drug Discov. Today Dis. Model* **2015**, *17–18*, 37–44. [CrossRef]
38. Divekar, R.; Kita, H. Recent advances in epithelium-derived cytokines (IL-33, IL-25, and thymic stromal lymphopoietin) and allergic inflammation. *Curr. Opin. Allergy Clin. Immunol.* **2015**, *15*, 98–103. [CrossRef] [PubMed]
39. Stark, J.M.; Tibbitt, C.A.; Coquet, J.M. The metabolic requirements of Th2 cell differentiation. *Front. Immunol.* **2019**, *10*, 2318. [CrossRef] [PubMed]
40. Humeniuk, P.; Dubiela, P.; Hoffmann-Sommergruber, K. Dendritic cells and their role in allergy: Uptake, proteolytic processing and presentation of allergens. *Int. J. Mol. Sci.* **2017**, *18*, 71491. [CrossRef]
41. Anvari, S.; Miller, J.; Yeh, C.Y.; Davis, C.M. IgE-Mediated Food Allergy. *Clin. Rev. Allergy Immunol.* **2019**, *57*, 244–260. [CrossRef]
42. Satitsuksanoa, P.; Daanje, M.; Akdis, M.; Boyd, S.D.; van de Veen, W. Biology and dynamics of B cells in the context of IgE-mediated food allergy. *Allergy Eur. J. Allergy Clin. Immunol.* **2021**, *76*, 1707–1717. [CrossRef]
43. Schoemaker, A.A.; Sprikkelman, A.B.; Grimshaw, K.E.; Roberts, G.; Grabenhenrich, L.; Rosenfeld, L.; Siegert, S.; Dubakiene, R.; Rudzeviciene, O.; Reche, M.; et al. Incidence and natural history of challenge-proven cow's milk allergy in European children—EuroPrevall birth cohort. *Allergy Eur. J. Allergy Clin. Immunol.* **2015**, *70*, 963–972. [CrossRef]
44. Flom, J.D.; Sicherer, S.H. Epidemiology of cow's milk allergy. *Nutrients* **2019**, *11*, 1051. [CrossRef]
45. Høst, A. Frequency of cow's milk allergy in childhood. *Ann. Allergy Asthma Immunol.* **2002**, *89*, 33–37. [CrossRef]
46. Hochwallner, H.; Schulmeister, U.; Swoboda, I.; Spitzauer, S.; Valenta, R. Cow's milk allergy: From allergens to new forms of diagnosis, therapy and prevention. *Methods* **2014**, *66*, 22–33. [CrossRef]
47. Walsh, J.; Meyer, R.; Shah, N.; Quekett, J.; Fox, A.T. Differentiating milk allergy (IgE and non-IgE mediated) from lactose intolerance: Understanding the underlying mechanisms and presentations. *Br. J. Gen. Pract.* **2016**, *66*, e609–e611. [CrossRef] [PubMed]
48. Fiocchi, A.; Dahdah, L.; Albarini, M.; Martelli, A. Cow's milk allergy in children and adults. *Chem. Immunol. Allergy* **2015**, *101*, 114–123. [PubMed]
49. Kalach, N.; Bellaïche, M.; Elias-Billon, I.; Dupont, C. Family history of atopy in infants with cow's milk protein allergy: A French population-based study. *Arch. Pediatr.* **2019**, *26*, 226–231. [CrossRef] [PubMed]
50. Haug, A.; Høstmark, A.T.; Harstad, O.M. Bovine milk in human nutrition—A review. *Lipids Health Dis.* **2007**, *6*, 25. [CrossRef]
51. Blanchard, E.; Zhu, P.; Schuck, P. Infant formula powders. In *Handbook of Food Powders: Processes and Properties*; Woodhead Publishing Limited: Sawston, UK, 2013. [CrossRef]
52. Malacarne, M.; Martuzzi, F.; Summer, A.; Mariani, P. Protein and fat composition of mare's milk: Some nutritional remarks with reference to human and cow's milk. *Int. Dairy J.* **2002**, *12*, 869–877. [CrossRef]
53. Bøgh, K.L.; Larsen, J.M. Chapter 19: Reducing allergenicity by proteolysis. In *Agents of Change: Enzymes in Milk and Dairy Products*; Springer: Berlin/Heidleberg, Germany, 2021; pp. 499–524. Available online: http://link.springer.com/10.1007/978-3-030-55482-8 (accessed on 15 January 2022).
54. Hurley, W.L.; Theil, P.K. Perspectives on immunoglobulins in colostrum and milk. *Nutrients* **2011**, *3*, 442–474. [CrossRef]
55. Pomés, A.; Davies, J.M.; Gadermaier, G.; Hilger, C.; Holzhauser, T.; Lidholm, J.; Lopata, A.L.; Mueller, G.A.; Nandy, A.; Radauer, C.; et al. WHO/IUIS Allergen Nomenclature: Providing a common language. *Mol. Immunol.* **2018**, *100*, 3–13. [CrossRef]
56. WHO/IUIS. Allergen Nomenclature Sub-Committee. Available online: http://allergen.org/ (accessed on 5 January 2022).
57. Smyth, E.; Clegg, R.A.; Holt, C. A biological perspective on the structure and function of caseins and casein micelles. *Int. J. Dairy Technol.* **2004**, *57*, 121–126. [CrossRef]
58. Holt, C.; Carver, J.A.; Ecroyd, H.; Thorn, D.C. Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods1. *J. Dairy Sci.* **2013**, *96*, 6127–6146. [CrossRef]
59. Holzmüller, W.; Gmach, O.; Griebel, A.; Kulozik, U. Casein precipitation by acid and rennet coagulation of buttermilk: Impact of pH and temperature on the isolation of milk fat globule membrane proteins. *Int. Dairy J.* **2016**, *63*, 115–123. [CrossRef]
60. Corredig, M.; Dalgleish, D.G. The mechanisms of the heat-induced interaction of whey proteins with casein micelles in milk. *Int. Dairy J.* **1999**, *9*, 233–236. [CrossRef]
61. Chapman, M. Allergen nomenclature. *Clin. Allergy Immunol.* **2008**, *21*, 47–58. [PubMed]
62. Monaci, L.; Tregoat, V.; van Hengel, A.J.; Anklam, E. Milk allergens, their characteristics and their detection in food: A review. *Eur. Food Res. Technol.* **2006**, *223*, 149–179. [CrossRef]
63. Vicente-Serrano, J.; Caballero, M.L.; Rodríguez-Pérez, R.; Carretero, P.; Pérez, R.; Blanco, J.G.; Juste, S.; Moneo, I. Sensitization to serum albumins in children allergic to cow's milk and epithelia. *Pediatr. Allergy Immunol.* **2007**, *18*, 503–507. [CrossRef]
64. Villa, C.; Costa, J.; Oliveira, M.B.P.P.; Mafra, I. Bovine Milk Allergens: A Comprehensive Review. *Compr. Rev. Food Sci. Food Saf.* **2018**, *17*, 137–164. [CrossRef]
65. Negaoui, H.; El Mecherfi, K.E.; Tadjer, S.A.; Grar, H.; Kheroua, O.; Saidi, D. Bovine lactoferrin allergenicity as studied in murine model of allergy. *Food Agric. Immunol.* **2016**, *27*, 711–723. [CrossRef]
66. Gaudin, J.C.; Rabesona, H.; Choiset, Y.; Yeretssian, G.; Chobert, J.M.; Sakanyan, V.; Drouet, M.; Haertlé, T. Assessment of the immunoglobulin E-mediated immune response to milk-specific proteins in allergic patients using microarrays. *Clin. Exp. Allergy* **2008**, *38*, 686–693. [CrossRef]

67. Natale, M.; Bisson, C.; Monti, G.; Peltran, A.; Garoffo, L.P.; Valentini, S.; Fabris, C.; Bertino, E.; Coscia, A.; Conti, A. Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Mol. Nutr. Food Res.* **2004**, *48*, 363–369. [CrossRef]
68. Van Regenmortel, M.H.V. What is a B-cell epitope? *Methods Mol. Biol.* **2009**, *524*, 3–20.
69. Arnon, R.; Van Regenmortel, M.H.V. Structural basis of antigenic specificity and design of new vaccines. *FASEB J.* **1992**, *6*, 3265–3274. [CrossRef]
70. Ansari, H.R.; Raghava, G.P. Identification of conformational B-cell Epitopes in an antigen from its primary sequence. *Immunome Res.* **2010**, *6*, 6. [CrossRef] [PubMed]
71. Miles, E.A.; Calder, P.C. Maternal diet and its influence on the development of allergic disease. *Clin. Exp. Allergy* **2015**, *45*, 63–74. [CrossRef] [PubMed]
72. Park, Y.H.; Kim, K.W.; Choi, B.S.; Jee, H.M.; Sohn, M.H.; Kim, K.E. Relationship between mode of delivery in childbirth and prevalence of allergic diseases in Korean children. *Allergy Asthma Immunol. Res.* **2010**, *2*, 28–33. [CrossRef] [PubMed]
73. McKeever, T.M.; Lewis, S.A.; Smith, C.; Hubbard, R. Mode of delivery and risk of developing allergic disease. *J. Allergy Clin. Immunol.* **2002**, *109*, 800–802. [CrossRef] [PubMed]
74. Kornienko, E.A. Probiotics and food allergy. *Vopr. Prakt. Pediatr.* **2016**, *11*, 60–64. [CrossRef]
75. Fox, A.; Bird, J.A.; Fiocchi, A.; Knol, J.; Meyer, R.; Salminen, S.; Sitang, G.; Szajewska, H.; Papadopoulos, N. The potential for pre-, pro- and synbiotics in the management of infants at risk of cow's milk allergy or with cow's milk allergy: An exploration of the rationale, available evidence and remaining questions. *World Allergy Organ. J.* **2019**, *12*, 100034. [CrossRef]
76. Vandenplas, Y. Prevention and management of cow's milk allergy in non-exclusively breastfed infants. *Nutrients* **2017**, *9*, 731. [CrossRef]
77. Du Toit, G.; Roberts, G.; Sayre, P.H.; Bahnson, H.T.; Radulovic, S.; Santos, A.F.; Brough, H.A.; Phippard, D.; Basting, M.; Feeney, M.; et al. Randomized Trial of Peanut Consumption in Infants at Risk for Peanut Allergy. *N. Engl. J. Med.* **2015**, *372*, 803–813. [CrossRef]
78. Perkin, M.R.; Logan, K.; Marrs, T.; Radulovic, S.; Craven, J.; Flohr, C.; Lack, G. Enquiring about Tolerance (EAT) study: Feasibility of an early allergenic food introduction regimen. *J. Allergy Clin. Immunol.* **2016**, *137*, 1477–1486.e8. [CrossRef]
79. Koplin, J.J.; Osborne, N.J.; Wake, M.; Martin, P.E.; Gurrin, L.C.; Robinson, M.N.; Tey, D.; Slaa, M.; Thiele, L.; Miles, L.; et al. Can early introduction of egg prevent egg allergy in infants? A population-based study. *J. Allergy Clin. Immunol.* **2010**, *126*, 807–813. [CrossRef]
80. American Academy of Pediatrics C of, N. Hypoallergenic Infant Formulas. *Pediatrics* **2000**, *106*, 346–349.
81. Halken, S.; Muraro, A.; de Silva, D.; Khaleva, E.; Angier, E.; Arasi, S.; Arshad, H.; Bahnson, H.T.; Beyer, K.; Boyle, R.; et al. EAACI guideline: Preventing the development of food allergy in infants and young children (2020 update). *Pediatr. Allergy Immunol.* **2021**, *32*, 843–858. [CrossRef] [PubMed]
82. Prescott, S.L.; Tang, M.L.K. The Australasian Society of Clinical Immunology and Allergy position statement: Summary of allergy prevention in children. *Med. J. Aust.* **2005**, *182*, 464–467. [CrossRef] [PubMed]
83. Joshi, P.A.; Smith, J.; Vale, S.; Campbell, D.E. The Australasian Society of Clinical Immunology and Allergy infant feeding for allergy prevention guidelines. *Med. J. Aust.* **2019**, *210*, 89–93. [CrossRef] [PubMed]
84. Fleischer, D.M.; Chan, E.S.; Venter, C.; Spergel, J.M.; Abrams, E.M.; Stukus, D.; Groetch, M.; Shaker, M.; Greenhawt, M. A Consensus Approach to the Primary Prevention of Food Allergy Through Nutrition: Guidance from the American Academy of Allergy, Asthma, and Immunology; American College of Allergy, Asthma, and Immunology; and the Canadian Society for Allergy and Clinical Immunology. *J. Allergy Clin. Immunol. Pract.* **2021**, *9*, 22–43.e4. [CrossRef]
85. Kramer, M.S.; Kakuma, R. Maternal dietary antigen avoidance during pregnancy or lactation, or both, for preventing or treating atopic disease in the child. *Evid.-Based Child Health* **2014**, *9*, 447–483. [CrossRef]
86. Tuokkola, J.; Luukkainen, P.; Tapanainen, H.; Kaila, M.; Vaarala, O.; Kenward, M.G.; Virta, L.J.; Veijola, R.; Simell, O.; Ilonen, J.; et al. Maternal diet during pregnancy and lactation and cow's milk allergy in offspring. *Eur. J. Clin. Nutr.* **2016**, *70*, 554–559. [CrossRef]
87. Stråvik, M.; Barman, M.; Hesselmar, B.; Sandin, A.; Wold, A.E.; Sandberg, A.S. Maternal intake of cow's milk during lactation is associated with lower prevalence of food allergy in offspring. *Nutrients* **2020**, *12*, 3680. [CrossRef]
88. Ewen, C. C-section babies are missing key microbes. *Nature* **2019**.
89. Selma-Royo, M.; Tarrazó, M.; García-Mantrana, I.; Gómez-Gallego, C.; Salminen, S.; Carmen Collado, M. Shaping Microbiota During the First 1000 Days of Life. In *Probiotics and Child Gastrointestinal Health*; Springer: Berlin/Heidelberg, Germany, 2019; pp. 3–24.
90. Lin, L.; Zhang, J. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. *BMC Immunol.* **2017**, *18*, 2. [CrossRef]
91. Adeyeye, T.E.; Yeung, E.H.; McLain, A.C.; Lin, S.; Lawrence, D.A.; Bell, E.M. Wheeze and Food Allergies in Children Born via Cesarean Delivery. *Am. J. Epidemiol.* **2019**, *188*, 355–362. [CrossRef] [PubMed]
92. Mitselou, N.; Hallberg, J.; Stephansson, O.; Almqvist, C.; Melén, E.; Ludvigsson, J.F. Cesarean delivery, preterm birth, and risk of food allergy: Nationwide Swedish cohort study of more than 1 million children. *J. Allergy Clin. Immunol.* **2018**, *142*, 1510–1514.e2. [CrossRef] [PubMed]
93. Pyrhönen, K.; Kulmala, P. Delivery mode and the incidence of atopic sensitization and food allergy in a Finnish child population. *Pediatr. Allergy Immunol.* **2021**, *33*, e13584. [CrossRef] [PubMed]
94. Rodgers, B.; Kirley, K.; Mounsey, A.; Ewigman, B. Prescribing an antibiotic? Pair it with probiotics. *J. Fam. Pract.* **2013**, *62*, 148–150.

95. Prescott, S.L.; Wiltschut, J.; Taylor, A.; Westcott, L.; Jung, W.; Currie, H.; Dunstan, J.A. Early markers of allergic disease in a primary prevention study using probiotics: 2.5-Year follow-up phase. *Allergy Eur. J. Allergy Clin. Immunol.* **2008**, *63*, 1481–1490. [CrossRef]
96. Plummer, E.L.; Chebar Lozinsky, A.; Tobin, J.M.; Uebergang, J.B.; Axelrad, C.; Garland, S.M.; Jacobs, S.E.; Tang, M.L.K. Postnatal probiotics and allergic disease in very preterm infants: Sub-study to the ProPrems randomized trial. *Allergy Eur. J. Allergy Clin. Immunol.* **2020**, *75*, 127–136. [CrossRef]
97. WHO. Infant and Young Child Feeding. 2021. Available online: https://www.who.int/news-room/fact-sheets/detail/infant-and-young-child-feeding#:~{}:text= (accessed on 2 February 2022).
98. Du Toit, G.; Katz, Y.; Sasieni, P.; Mesher, D.; Maleki, S.J.; Fisher, H.R.; Fox, A.T.; Turcanu, V.; Amir, T.; Zadik-Mnuhin, G.; et al. Early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy. *J. Allergy Clin. Immunol.* **2008**, *122*, 984–991. [CrossRef]
99. Yakaboski, E.; Robinson, L.B.; Arroyo, A.; Espinola, J.A.; Geller, R.J.; Sullivan, A.F.; Rudders, S.A.; Camargo, C.A. Early Introduction of Food Allergens and Risk of Developing Food Allergy. *Nutrients* **2021**, *20*, 216. [CrossRef]
100. Abrams, E.M.; Sicherer, S.H. Cow's milk allergy prevention. *Ann. Allergy Asthma Immunol.* **2021**, *127*, 36–41. [CrossRef]
101. Katz, Y.; Rajuan, N.; Goldberg, M.R.; Eisenberg, E.; Heyman, E.; Cohen, A.; Leshno, M. Early exposure to cow's milk protein is protective against IgE-mediated cow's milk protein allergy. *J. Allergy Clin. Immunol.* **2010**, *126*, 77–82.e1. [CrossRef]
102. Chan, E.S.; Abrams, E.M.; Hildebrand, K.J.; Watson, W. Early introduction of foods to prevent food allergy. *Allergy Asthma Clin. Immunol.* **2018**, *14*, 57. [CrossRef] [PubMed]
103. Osborn, D.A.; Sinn, J.K.H.; Jones, L.J. Infant formulas containing hydrolysed protein for prevention of allergic disease. *Cochrane Database Syst Rev.* **2018**, *2018*, 1–119. [CrossRef] [PubMed]
104. Koletzko, S.; Niggemann, B.; Arato, A.; Dias, J.A.; Heuschkel, R.; Husby, S.; Mearin, M.L.; Papadopoulou, A.; Ruemmele, F.M.; Staiano, A.; et al. Diagnostic approach and management of cow's-milk protein allergy in infants and children: Espghan gi committee practical guidelines. *J. Pediatr. Gastroenterol. Nutr.* **2012**, *55*, 221–229. [CrossRef] [PubMed]
105. Luyt, D.; Ball, H.; Makwana, N.; Green, M.R.; Bravin, K.; Nasser, S.M.; Clark, A.T. BSACI guideline for the diagnosis and management of cow's milk allergy. *Clin. Exp. Allergy* **2014**, *44*, 642–672. [CrossRef] [PubMed]
106. Fiocchi, A.; Brozek, J.; Schu, H.; Von Berg, A.; Beyer, K.; Bozzola, M.; Bradsher, J.; Compalati, E.; Ebisawa, M.; Guzman, M.A.; et al. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. *Pediatr. Allergy Immunol.* **2010**, *21*, 1–125. [CrossRef]
107. Vandenplas, Y.; Brough, H.A.; Fiocchi, A.; Miqdady, M.; Munasir, Z.; Salvatore, S.; Thapar, N.; Venter, C.; Vieira, M.C.; Meyer, R. Current Guidelines and Future Strategies for the Management of Cow's Milk Allergy. *J. Asthma Allergy* **2021**, *14*, 1243–1256. [CrossRef]
108. Vandenplas, Y.; Brueton, M.; Dupont, C.; Hill, D.; Isolauri, E.; Koletzko, S.; Oranje, A.P.; Staiano, A. Guidelines for the diagnosis and management of cow's milk protein allergy in infants. *Arch. Dis. Child.* **2007**, *92*, 902–908. [CrossRef]
109. Høst, A.; Husby, S.; Hansen, L.G.; Østerballe, O. Bovine β-lactoglobulin in human milk from atopic and non-atopic mothers. Relationship to maternal intake of homogenized and unhomogenized milk. *Clin. Exp. Allergy* **1990**, *20*, 383–387. [CrossRef]
110. Ludman, S.; Shah, N.; Fox, A.T. Managing cows' milk allergy in children. *BMJ* **2013**, *347*, f5424. [CrossRef]
111. Chruszcz, M.; Mikolajczak, K.; Mank, N.; Majorek, K.A.; Porebski, P.J.; Minor, W. Serum albumins—Unusual allergens. *Biochim. Biophys. Acta-Gen. Subj.* **2013**, *1830*, 5375–5381. [CrossRef]
112. WHO. *Codex Alimentarius: Standard for Infant Formula and Formulas For Special Medical Purposes Intended For Infants*; World Health Organization: Geneva, Switzerland, 2015.
113. Sharif, M.K.; Zahid, A.; Shah, F.-u.-H. Role of Food Product Development in Increased Food Consumption and Value Addition. In *Food Processing for Increased Quality and Consumption*; Elsevier Inc.: Amsterdam, The Netherlands, 2018. [CrossRef]
114. EU Commission. Regulations. Commission delegated regulation (EU) 2016/127. *Off. J. Eur. Union* **2016**, *L25*, 1–29.
115. WHO/UNICEF. *International Code for Marketing Breastmilk Substitutes*; WHO: Geneva, Switzerland, 1981.
116. Koletzko, B.; Beyer, J.; Brands, B.; Demmelmair, H.; Grote, V.; Haile, G.; Gruszfeld, D.; Rzehak, P.; Socha, P.; Weber, M. Early influences of nutrition on postnatal growth. *Nestle Nutr. Inst. Workshop Ser.* **2013**, *71*, 11–27.
117. Leung, A.K.C.; Sauve, R.S. Whole cow's milk in infancy. *Paediatr. Child Health* **2003**, *8*, 419–421. [CrossRef] [PubMed]
118. Bhatia, J.; Greer, F. Use of soy protein-based formulas in infant feeding. *Pediatrics* **2008**, *121*, 1062–1068. [CrossRef] [PubMed]
119. Restani, P.; Ballabio, C.; Di Lorenzo, C.; Tripodi, S.; Fiocchi, A. Molecular aspects of milk allergens and their role in clinical events. *Anal. Bioanal. Chem.* **2009**, *395*, 47–56. [CrossRef]
120. Ye, A.; Cui, J.; Carpenter, E.; Prosser, C.; Singh, H. Dynamic in vitro gastric digestion of infant formulae made with goat milk and cow milk: Influence of protein composition. *Int. Dairy J.* **2019**, *97*, 76–85. [CrossRef]
121. Jia, K.; Feng, Y.; Brenna, J.T.; Luo, Z.C.; Zhao, J.; Li, H.; Li, P.; Zhang, Q.; Zhao, Q.; Dai, Q.; et al. Breast milk EPA associated with infant distractibility when EPA level is low. *Nutrition* **2021**, *86*, 111143. [CrossRef] [PubMed]
122. Horrocks, L.A.; Yeo, Y.K. Health benefits of docosahexaenoic acid (DHA). *Pharmacol. Res.* **1999**, *40*, 211–225. [CrossRef]
123. Tallima, H.; El Ridi, R. Arachidonic acid: Physiological roles and potential health benefits—A review. *J. Adv. Res.* **2018**, *11*, 33–41. [CrossRef]
124. Bryś, J.; Górska, A.; Ostrowska-Ligęza, E.; Wirkowska-Wojdyła, M.; Bryś, A.; Brzezińska, R.; Dolatowska-żebrowska, K.; Małajowicz, J.; Ziarno, M.; Obranović, M.; et al. Human milk fat substitutes from lard and hemp seed oil mixtures. *Appl. Sci.* **2021**, *11*, 7014. [CrossRef]

125. Parschat, K.; Melsaether, C.; Jäpelt, K.R.; Jennewein, S. Clinical evaluation of 16-week supplementation with 5HMO-mix in healthy-term human infants to determine tolerability, safety, and effect on growth. *Nutrients* **2021**, *13*, 2871. [CrossRef]
126. Bakker-Zierikzee, A.M.; Alles, M.S.; Knol, J.; Kok, F.J.; Tolboom, J.J.M.; Bindels, J.G. Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable Bifidobacterium animalis on the intestinal microflora during the first 4 months of life. *Br. J. Nutr.* **2005**, *94*, 783–790. [CrossRef] [PubMed]
127. Pehrsson, P.R.; Patterson, K.Y.; Khan, M.A. Selected vitamins, minerals and fatty acids in infant formulas in the United States. *J. Food Compos. Anal.* **2014**, *36*, 66–71. [CrossRef]
128. Green Corkins, K.; Shurley, T. What's in the Bottle? A Review of Infant Formulas. *Nutr. Clin. Pract.* **2016**, *31*, 723–729. [CrossRef] [PubMed]
129. Roy, D.; Ye, A.; Moughan, P.J.; Singh, H. Composition, Structure, and Digestive Dynamics of Milk From Different Species—A Review. *Front. Nutr.* **2020**, *7*, 195. [CrossRef] [PubMed]
130. Guo, M.; Ahmad, S. Formulation guidelines for infant formula. In *Human Milk Biochemistry and Infant Formula Manufacturing Technology*; Woodhead Publishing Limited: Sawston, UK, 2014. [CrossRef]
131. Happe, R.P.; Gambelli, L. Infant formula. In *Specialty Oils and Fats in Food and Nutrition: Properties, Processing and Applications*; Elsevier Ltd.: Amsterdam, The Netherlands, 2015. [CrossRef]
132. Hochwallner, H.; Schulmeister, U.; Swoboda, I.; Focke-Tejkl, M.; Reininger, R.; Civaj, V.; Campana, R.; Thalhamer, J.; Scheiblhofer, S.; Balic, N.; et al. Infant milk formulas differ regarding their allergenic activity and induction of T-cell and cytokine responses. *Allergy Eur. J. Allergy Clin. Immunol.* **2017**, *72*, 416–424. [CrossRef]
133. Fenelon, M.A.; Hickey, R.M.; Buggy, A.; McCarthy, N.; Murphy, E.G. Whey proteins in infant formula. In *Whey Proteins: From Milk to Medicine*; Elsevier Inc.: Amsterdam, The Netherlands, 2018; pp. 439–494. [CrossRef]
134. Golkar, A.; Milani, J.M.; Vasiljevic, T. Altering allergenicity of cow's milk by food processing for applications in infant formula. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 159–172. [CrossRef]
135. Host, A.; Halken, S. Hypoallergenic formulas—When, to whom and how long: After more than 15 years we know the right indication! *Allergy* **2004**, *59*, 45–52. [CrossRef]
136. Jiang, Y.J.; Guo, M. Processing technology for infant formula. In *Human Milk Biochemistry and Infant Formula Manufacturing Technology*; Woodhead Publishing Limited: Sawston, UK, 2014. [CrossRef]
137. Bu, G.; Luo, Y.; Chen, F.; Liu, K.; Zhu, T. Milk processing as a tool to reduce cow's milk allergenicity: A mini-review. *Dairy Sci. Technol.* **2013**, *93*, 211–223. [CrossRef]
138. Fischer, J. Keeping enzymes kosher: Sacred and secular biotech production. *EMBO Rep.* **2015**, *16*, 681–684. [CrossRef]
139. Morisawa, Y.; Kitamura, A.; Ujihara, T.; Zushi, N.; Kuzume, K.; Shimanouchi, Y.; Tamura, S.; Wakiguchi, H.; Saito, H.; Matsumoto, K. Effect of heat treatment and enzymatic digestion on the B cell epitopes of cow's milk proteins. *Clin. Exp. Allergy* **2009**, *39*, 918–925. [CrossRef] [PubMed]
140. Vandenplas, Y.; Bhatia, J.; Shamir, R.; Agostoni, C.; Turck, D.; Staiano, A.; Szajewska, H. Hydrolyzed formulas for allergy prevention. *J. Pediatr. Gastroenterol. Nutr.* **2014**, *58*, 549–552. [CrossRef]
141. Lowe, A.J.; Dharmage, S.C.; Allen, K.J.; Tang, M.L.; Hill, D.J. The role of partially hydrolyzed whey formula for the prevention of allergic disease: Evidence and gaps. *Expert Rev. Clin. Immunol.* **2013**, *9*, 31–41. [CrossRef] [PubMed]
142. Meulenbroek, L.A.P.M.; Den Hartog Jager, C.F.; Lebens, A.F.M.; Knulst, A.C.; Bruijnzeel-Koomen, C.A.F.M.; Garssen, J.; Knippels, L.M.J.; Van Hoffen, E. Characterization of T cell epitopes in bovine α-lactalbumin. *Int. Arch. Allergy Immunol.* **2014**, *163*, 292–296. [CrossRef] [PubMed]
143. Fiocchi, A.; Schünemann, H.J.; Brozek, J.; Restani, P.; Beyer, K.; Troncone, R.; Martelli, A.; Terracciano, L.; Bahna, S.L.; Rancé, F.; et al. Diagnosis and rationale for action against Cow's milk allergy (DRACMA): A summary report. *J. Allergy Clin. Immunol.* **2010**, *126*, 1119–1128. [CrossRef]
144. Verduci, E.; D'Elios, S.; Cerrato, L.; Comberiati, P.; Calvani, M.; Palazzo, S.; Martelli, A.; Landi, M.; Trikamjee, T.; Peroni, D.G. Cow's Milk Substitutes for Children: Nutritional Aspects of Milk from Different Mammalian Species, Special Formula and Plant-Based Beverages. *Nutrients* **2019**, *11*, 1739. [CrossRef]
145. Vandenplas, Y.; Munasir, Z.; Hegar, B.; Kumarawati, D.; Suryawan, A.; Kadim, M.; Djais, J.T.; Basrowi, R.W.; Krisnamurti, D. A perspective on partially hydrolyzed protein infant formula in nonexclusively breastfed infants. *Korean J. Pediatr.* **2019**, *62*, 149–154. [CrossRef]
146. Niggemann, B.; Binder, C.; Klettke, U.; Wahn, U. In Vivo and In Vitro studies on the residual allergenicity of partially hydrolysed infant formulae. *Acta Paediatr. Nurtur. Child* **1999**, *88*, 394–398. [CrossRef]
147. Caffarelli, C.; Plebani, A.; Poiesi, C.; Petroccione, T.; Spattini, A.; Cavagni, G. Determination of allergenicity to three cow's milk hydrolysates and an amino acidderived formula in children with cow's milk allergy. *Clin. Exp. Allergy* **2002**, *32*, 74–79. [CrossRef]
148. Van Esch, B.C.A.M.; van Bilsen, J.H.M.; Jeurink, P.V.; Garssen, J.; Penninks, A.H.; Smit, J.J.; Pieters, R.H.H.; Knippels, L.M.J. Interlaboratory evaluation of a cow's milk allergy mouse model to assess the allergenicity of hydrolysed cow's milk based infant formulas. *Toxicol. Lett.* **2013**, *220*, 95–102. [CrossRef]
149. Van Esch, B.C.A.M.; van Bilsen, J.H.M.; Gros-van Hest, M.; Kleinjans, L.; Belzer, C.; Jeurink, P.V.; Garssen, J.; Smit, J.J.; Pieters, R.H.H.; Knippels, L.M.J. A multi-center assessment to compare residual allergenicity of partial hydrolyzed whey proteins in a murine model for cow's milk allergy—Comparison to the single parameter guinea pig model. *Toxicol. Lett.* **2020**, *333*, 312–321. [CrossRef] [PubMed]

150. Fritsche, R. Animal models in food allergy: Assessment of allergenicity and preventive activity of infant formulas. *Toxicol. Lett.* **2003**, *141*, 303–309. [CrossRef]
151. Jensen, L.H.; Larsen, J.M.; Madsen, C.B.; Laursen, R.R.; Jacobsen, L.N.; Bøgh, K.L. Preclinical Brown Norway Rat Models for the Assessment of Infant Formulas in the Prevention and Treatment of Cow's Milk Allergy. *Int. Arch. Allergy Immunol.* **2019**, *178*, 307–314. [CrossRef] [PubMed]
152. Ahrens, B.; Quarcoo, D.; Buhner, S.; Reese, G.; Vieths, S.; Hamelmann, E. Development of an animal model to evaluate the allergenicity of food allergens. *Int. Arch. Allergy Immunol.* **2014**, *164*, 89–96. [CrossRef]
153. Bøgh, K.L.; Barkholt, V.; Madsen, C.B. Characterization of the Immunogenicity and Allergenicity of Two Cow's Milk Hydrolysates—A Study in Brown Norway Rats. *Scand. J. Immunol.* **2015**, *81*, 274–283. [CrossRef]
154. Liang, X.; Wang, Z.; Yang, H.; Luo, X.; Sun, J.; Yang, M.; Shi, X.; Yue, X.; Zheng, Y. Evaluation of allergenicity of cow milk treated with enzymatic hydrolysis through a mouse model of allergy. *J. Dairy Sci.* **2022**, *105*, 1039–1050. [CrossRef]
155. Van Esch, B.C.A.M.; Knipping, K.; Jeurink, P.; van der Heide, S.; Dubois, A.E.J.; Willemsen, L.E.M.; Garssen, J.; Knippels, L.M.J. In vivo and in vitro evaluation of the residual allergenicity of partially hydrolysed infant formulas. *Toxicol. Lett.* **2011**, *201*, 264–269. [CrossRef]
156. Kiewiet, M.B.G.; van Esch, B.C.A.M.; Garssen, J.; Faas, M.M.; de Vos, P. Partially hydrolyzed whey proteins prevent clinical symptoms in a cow's milk allergy mouse model and enhance regulatory T and B cell frequencies. *Mol. Nutr. Food Res.* **2017**, *61*, 1700340. [CrossRef]
157. Vandenplas, Y.; Hauser, B.; den Borre, C.; Sacre, L.; Dab, I. Effect of a whey hydrolysate prophylaxis of atopic disease. *Ann. Allergy* **1992**, *68*, 419–424.
158. Chandra, R.K. Five-year follow up of high-risk infants with family history of allergy exclusively breast-fed or fed partial whey hydrolysate, soy and conventional cow's milk formulas. *Nutr. Res.* **1998**, *18*, 1395–1411. [CrossRef]
159. Von Berg, A.; Filipiak-Pittroff, B.; Krämer, U.; Link, E.; Heinrich, J.; Koletzko, S.; Grübl, A.; Hoffmann, U.; Beckmann, C.; Reinhardt, D.; et al. The German Infant Nutritional Intervention Study (GINI) for the preventive effect of hydrolysed infant formulas in infants at high risk for allergic diseases. Design and selected results. *Allergol. Sel.* **2017**, *1*, 28–39. [CrossRef] [PubMed]
160. Lowe, A.J.; Hosking, C.S.; Bennett, C.M.; Allen, K.J.; Axelrad, C.; Carlin, J.B.; Abramson, M.J.; Dharmage, S.C.; Hill, D.J. Effect of a partially hydrolyzed whey infant formula at weaning on risk of allergic disease in high-risk children: A randomized controlled trial. *J. Allergy Clin. Immunol.* **2011**, *128*, 360–365.e4. [CrossRef] [PubMed]
161. De Silva, D.; Halken, S.; Singh, C.; Muraro, A.; Angier, E.; Arasi, S.; Arshad, H.; Beyer, K.; Boyle, R.; du Toit, G.; et al. Preventing food allergy in infancy and childhood: Systematic review of randomised controlled trials. *Pediatr. Allergy Immunol.* **2020**, *31*, 813–826. [CrossRef] [PubMed]
162. Graversen, K.B.; Larsen, J.M.; Pedersen, S.S.; Sørensen, L.V.; Christoffersen, H.F.; Jacobsen, L.N.; Halken, S.; Licht, T.R.; Bahl, M.I.; Bøgh, K.L. Partially Hydrolysed Whey Has Superior Allergy Preventive Capacity Compared to Intact Whey Regardless of Amoxicillin Administration in Brown Norway Rats. *Front. Immunol.* **2021**, *12*, 705543. [CrossRef] [PubMed]
163. Fritsché, R.; Pahud, J.J.; Pecquet, S.; Pfeifer, A. Induction of systemic immunologic tolerance to β-lactoglobulin by oral administration of a whey protein hydrolysate. *J. Allergy Clin. Immunol.* **1997**, *100*, 266–273. [CrossRef]
164. Chikhi, A.; Elmecherfi, K.E.; Bernard, H.; Cortes-Perez, N.; Kheroua, O.; Saidi, D.; Adel-Patient, K. Evaluation of the efficiency of hydrolyzed whey formula to prevent cow's milk allergy in the BALB/c mouse model. *Pediatr. Allergy Immunol.* **2019**, *30*, 370–377. [CrossRef]
165. Lambers, T.T.; Gloerich, J.; van Hoffen, E.; Alkema, W.; Hondmann, D.H.; van Toll, E.A.F. Clustering analyses in peptidomics revealed that peptide profiles of infant formulae are descriptive. *Food Sci. Nutr.* **2015**, *3*, 81–90. [CrossRef]
166. Rosendal, A.; Barkholt, V. Detection of Potentially Allergenic Material in 12 Hydrolyzed Milk Formulas. *J. Dairy Sci.* **2000**, *83*, 2200–2210. [CrossRef]
167. Gomes-Santos, A.C.; Fonseca, R.C.; Lemos, L.; Reis, D.S.; Moreira, T.G.; Souza, A.L.; Silva, M.R.; Silvestre, M.P.C.; Cara, D.C.; Faria, A.M.C. Hydrolyzed whey protein prevents the development of food allergy to β-lactoglobulin in sensitized mice. *Cell Immunol.* **2015**, *298*, 47–53. [CrossRef]
168. Van Beresteijn, E.C.H.; Meijer, R.J.G.M.; Schmidt, D.G. Residual antigenicity of hypoallergenic infant formulas and the occurrence of milk-specific IgE antibodies in patients with clinical allergy. *J. Allergy Clin. Immunol.* **1995**, *96*, 365–374. [CrossRef]
169. Schwartz, R.H.; Amonette, M.S. Cow milk protein hydrolysate infant formulas not always "hypoallergenic". *J. Pediatr.* **1991**, *119*, 839–840. [CrossRef]
170. Miraglia Del Giudice, M.; D'Auria, E.; Peroni, D.; Palazzo, S.; Radaelli, G.; Comberiati, P.; Galdo, F.; Maiello, N.; Riva, E. Flavor, relative palatability and components of cow's milk hydrolysed formulas and amino acid-based formula Allergology and Immunology. *Ital. J. Pediatr.* **2015**, *41*, 42. [CrossRef] [PubMed]
171. Liu, X.; Jiang, D.; Peterson, D.G. Identification of bitter peptides in whey protein hydrolysate. *J. Agric. Food Chem.* **2014**, *62*, 5719–5725. [CrossRef] [PubMed]
172. Granier, A.; Goulet, O.; Hoarau, C. Fermentation products: Immunological effects on human and animal models. *Pediatr. Res.* **2013**, *74*, 238–244. [CrossRef] [PubMed]
173. Pescuma, M.; Hébert, E.M.; Mozzi, F.; De Valdez, G.F. Hydrolysis of whey proteins by Lactobacillus acidophilus, Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus grown in a chemically defined medium. *J. Appl. Microbiol.* **2007**, *103*, 1738–1746. [CrossRef] [PubMed]

174. El-Ghaish, S.; Rabesona, H.; Choiset, Y.; Sitohy, M.; Haertlé, T.; Chobert, J.M. Proteolysis by Lactobacillus fermentum IFO3956 isolated from Egyptian milk products decreases immuno-reactivity of αs1-casein. *J. Dairy Res.* **2011**, *78*, 203–210. [CrossRef]
175. Ahmadova, A.; El-Ghaish, S.; Choiset, Y.; Rabesona, H.; Drouet, M.; Chobert, J.M.; Kuliev, A.A.; Haertle, T. Modification of ige binding to β- and αs1-caseins by proteolytic activity of lactobacillus helveticus a75. *J. Food Biochem.* **2013**, *37*, 491–500. [CrossRef]
176. Pescuma, M.; Hébert, E.M.; Rabesona, H.; Drouet, M.; Choiset, Y.; Haertlé, T.; Mozzi, F.; De Valdez, G.F.; Chobert, J.M. Proteolytic action of Lactobacillus delbrueckii subsp. bulgaricus CRL 656 reduces antigenic response to bovine β-lactoglobulin. *Food Chem.* **2011**, *127*, 487–492. [CrossRef]
177. Ménard, S.; Candalh, C.; Ben, A.M.; Rakotobe, S.; Gaboriau-Routhiau, V.; Cerf-Bensussan, N.; Heyman, M. Stimulation of immunity without alteration of oral tolerance in mice fed with heat-treated fermented infant formula. *J. Pediatr. Gastroenterol. Nutr.* **2006**, *43*, 451–458. [CrossRef]
178. Morisset, M.; Aubert-Jacquin, C.; Soulaines, P.; Moneret-Vautrin, D.A.; Dupont, C. A non-hydrolyzed, fermented milk formula reduces digestive and respiratory events in infants at high risk of allergy. *Eur. J. Clin. Nutr.* **2011**, *65*, 175–183. [CrossRef]
179. Szajewska, H.; Skórka, A.; Pieścik-Lech, M. Fermented infant formulas without live bacteria: A systematic review. *Eur. J. Pediatr.* **2015**, *174*, 1413–1420. [CrossRef] [PubMed]
180. Nasirpour, A.; Scher, J.; Desobry, S. Baby foods: Formulations and interactions (a review). *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 665–681. [CrossRef] [PubMed]
181. Pischetsrieder, M.; Henle, T. Glycation products in infant formulas: Chemical, analytical and physiological aspects. *Amino Acids* **2012**, *42*, 1111–1118. [CrossRef] [PubMed]
182. Rudloff, S.; Lonnerdal, B. Solubility and Digestability of Milk Proteins in Infant Formulas Exposed to Different Heat Treatments. *J. Pediatr. Gastroenterol. Nutr.* **1992**, *15*, 25–33. [CrossRef]
183. De Wit, J.N. Nutritional and Functional Characteristics of Whey Proteins in Food Products. *J. Dairy Sci.* **1998**, *81*, 597–608. [CrossRef]
184. De Witt, J.N.; Klarenbeek, G. Effects of Various Heat Treatments on Structure and Solubility of Whey Proteins. *J. Dairy Sci.* **1984**, *67*, 2701–2710. [CrossRef]
185. Donato, L.; Guyomarc'h, F. Formation and properties of the whey protein/κ-casein complexes in heated skim milk—A review. *Dairy Sci. Technol.* **2009**, *89*, 3–29. [CrossRef]
186. Van Lieshout, G.A.A.; Lambers, T.T.; Bragt, M.C.E.; Hettinga, K.A. How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2422–2445. [CrossRef]
187. Guyomarc'h, F.; Law, A.J.R.; Dalgleish, D.G. Formation of soluble and micelle-bound protein aggregates in heated milk. *J. Agric. Food Chem.* **2003**, *51*, 4652–4660. [CrossRef]
188. Lowe, E.K.; Anema, S.G.; Bienvenue, A.; Roland, M.J.; Creamer, L.K.; Jiménez-Flores, R. Heat-induced redistribution of disultide bonds in milk proteins. 2. Disulfide bonding patterns between bovine β-lactoglobulin and κ-casein. *J. Agric. Food Chem.* **2004**, *52*, 7669–7680. [CrossRef]
189. Creamer, L.K.; Bienvenue, A.; Nilsson, H.; Paulsson, M.; Van Wanroij, M.; Lowe, E.K.; Anema, S.G.; Boland, M.J.; Jiménez-Flores, R. Heat-induced redistribution of disulfide bonds in milk proteins. 1. Bovine β-lactoglobulin. *J. Agric. Food Chem.* **2004**, *52*, 7660–7668. [CrossRef] [PubMed]
190. Krishna, T.C.; Najda, A.; Bains, A.; Tosif, M.M.; Papliński, R.; Kapłan, M.; Chawla, P. Influence of ultra-heat treatment on properties of milk proteins. *Polymers* **2021**, *13*, 3164. [CrossRef] [PubMed]
191. Corredig, M.; Dalgleish, D.G. Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. *Food Res. Int.* **1996**, *29*, 49–55. [CrossRef]
192. Dalgleish, D.G.; Corredig, M. The structure of the casein micelle of milk and its changes during processing. *Annu. Rev. Food Sci. Technol.* **2012**, *3*, 449–467. [CrossRef]
193. Singh, H.; Latham, J.M. Heat stability of milk: Aggregation and dissociation of protein at ultra-high temperatures. *Int. Dairy J.* **1993**, *3*, 225–237. [CrossRef]
194. Donovan, M.; Mulvihill, D.M. Thermal Denaturation and Aggregation of Whey Proteins. *Agric. Food Dev. Auth.* **1987**, *11*, 87–100.
195. Chaplin, L.C.; Lyster, R.L. Irreversible heat denaturation of bovine alpha-lactalbumin. *J. Dairy Res.* **1986**, *53*, 249–258. [CrossRef]
196. Nguyen, N.H.A.; Streicher, C.; Anema, S.G. The effect of thiol reagents on the denaturation of the whey protein in milk and whey protein concentrate solutions. *Int. Dairy J.* **2018**, *85*, 285–293. [CrossRef]
197. Abbring, S.; Xiong, L.; Diks, M.A.P.; Baars, T.; Garssen, J.; Hettinga, K.; Van Esch, B.C.A.M. Loss of allergy-protective capacity of raw cow's milk after heat treatment coincides with loss of immunologically active whey proteins. *Food Funct.* **2020**, *11*, 4982–4993. [CrossRef]
198. Ye, M.P.; Zhou, R.; Shi, Y.R.; Chen, H.C.; Du, Y. Effects of heating on the secondary structure of proteins in milk powders using mid-infrared spectroscopy. *J. Dairy Sci.* **2017**, *100*, 89–95. [CrossRef]
199. Crowley, S.V.; Dowling, A.P.; Caldeo, V.; Kelly, A.L.; O'Mahony, J.A. Impact of α-lactalbumin:β-lactoglobulin ratio on the heat stability of model infant milk formula protein systems. *Food Chem.* **2016**, *194*, 184–190. [CrossRef] [PubMed]
200. Halabi, A.; Deglaire, A.; Hamon, P.; Bouhallab, S.; Dupont, D.; Croguennec, T. Kinetics of heat-induced denaturation of proteins in model infant milk formulas as a function of whey protein composition. *Food Chem.* **2020**, *302*, 125296. [CrossRef] [PubMed]
201. Dupont, D.; Boutrou, R.; Menard, O.; Jardin, J.; Tanguy, G.; Schuck, P.; Haab, B.B.; Leonil, J. Heat treatment of milk during powder manufacture increases casein resistance to simulated infant digestion. *Food Dig.* **2010**, *1*, 28–39. [CrossRef]

202. He, T.; Rombouts, W.; Einerhand, A.W.C.; Hotrum, N.; van de Velde, F. Gastric protein digestion of goat and cow milk infant formula and human milk under simulated infant conditions. *Int. J. Food Sci. Nutr.* **2021**, *73*, 28–38. [CrossRef]
203. Halabi, A.; Croguennec, T.; Bouhallab, S.; Dupont, D.; Deglaire, A. Modification of protein structures by altering the whey protein profile and heat treatment affects: In vitro static digestion of model infant milk formulas. *Food Funct.* **2020**, *11*, 6933–6945. [CrossRef]
204. Rinaldi, L.; Gauthier, S.F.; Britten, M.; Turgeon, S.L. Invitro gastrointestinal digestion of liquid and semi-liquid dairy matrixes. *LWT-Food Sci. Technol.* **2014**, *57*, 99–105. [CrossRef]
205. Barbé, F.; Ménard, O.; Le Gouar, Y.; Buffière, C.; Famelart, M.H.; Laroche, B.; Le Feunteun, S.; Dupont, D.; Rémond, D. The heat treatment and the gelation are strong determinants of the kinetics of milk proteins digestion and of the peripheral availability of amino acids. *Food Chem.* **2013**, *136*, 1203–1212. [CrossRef]
206. Peram, M.R.; Loveday, S.M.; Ye, A.; Singh, H. In Vitro gastric digestion of heat-induced aggregates of β-lactoglobulin. *J. Dairy Sci.* **2013**, *96*, 63–74. [CrossRef]
207. Meltretter, J.; Seeber, S.; Humeny, A.; Becker, C.M.; Pischetsrieder, M. Site-specific formation of maillard, oxidation, and condensation products from whey proteins during reaction with lactose. *J. Agric. Food Chem.* **2007**, *55*, 6096–6103. [CrossRef]
208. Chen, Z.; Leinisch, F.; Greco, I.; Zhang, W.; Shu, N.; Chuang, C.Y.; Lund, M.N.; Davies, M.J. Characterisation and quantification of protein oxidative modifications and amino acid racemisation in powdered infant milk formula. *Free Radic. Res.* **2019**, *53*, 68–81. [CrossRef]
209. Fenaille, F.; Parisod, V.; Tabet, J.C.; Guy, P.A. Carbonylation of milk powder proteins as a consequence of processing conditions. *Proteomics* **2005**, *5*, 3097–3104. [CrossRef]
210. Fenaille, F.; Parisod, V.; Visani, P.; Populaire, S.; Tabet, J.C.; Guy, P.A. Modifications of milk constituents during processing: A preliminary benchmarking study. *Int. Dairy J.* **2006**, *16*, 728–739. [CrossRef]
211. Stadtman, E.R.; Levine, R.L. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* **2003**, *25*, 207–218. [CrossRef] [PubMed]
212. Scheidegger, D.; Radici, P.M.; Vergara-Roig, V.A.; Bosio, N.S.; Pesce, S.F.; Pecora, R.P.; Romano, J.C.P.; Kivatinitz, S.C. Evaluation of milk powder quality by protein oxidative modifications. *J. Dairy Sci.* **2013**, *96*, 3414–3423. [CrossRef] [PubMed]
213. Giblin, L.; Yalçın, A.S.; Biçim, G.; Krämer, A.C.; Chen, Z.; Callanan, M.J.; Arranz, E.; Davies, M.J. Whey proteins: Targets of oxidation, or mediators of redox protection. *Free Radic. Res.* **2019**, *53*, 1136–1152. [CrossRef]
214. Davies, M.J. Protein oxidation and peroxidation. *Biochem. J.* **2016**, *473*, 805–825. [CrossRef] [PubMed]
215. Feng, X.; Li, C.; Ullah, N.; Cao, J.; Lan, Y.; Ge, W.; Hackman, R.M.; Li, Z.; Chen, L. Susceptibility of whey protein isolate to oxidation and changes in physicochemical, structural, and digestibility characteristics. *J. Dairy Sci.* **2015**, *98*, 7602–7613. [CrossRef]
216. Chang, C.H.; Zhao, X.H. In vitro digestibility and rheological properties of caseinates treated by an oxidative system containing horseradish peroxidase, glucose oxidase and glucose. *Int. Dairy J.* **2012**, *27*, 47–52. [CrossRef]
217. Meyer, B.; Baum, F.; Vollmer, G.; Pischetsrieder, M. Distribution of protein oxidation products in the proteome of thermally processed milk. *J. Agric. Food Chem.* **2012**, *60*, 7306–7311. [CrossRef]
218. Gill, V.; Kumar, V.; Singh, K.; Kumar, A.; Kim, J.J. Advanced glycation end products (AGEs) may be a striking link between modern diet and health. *Biomolecules* **2019**, *9*, 888. [CrossRef]
219. Marvin, L.F.; Parisod, V.; Fay, L.B.; Guy, P.A. Characterization of lactosylated proteins of infant formula powders using two-dimensional gel electrophoresis and nanoelectrospray mass spectrometry. *Electrophoresis* **2002**, *23*, 2505–2512. [CrossRef]
220. Milkovska-Stamenova, S.; Hoffmann, R. Influence of storage and heating on protein glycation levels of processed lactose-free and regular bovine milk products. *Food Chem.* **2017**, *221*, 489–495. [CrossRef] [PubMed]
221. Wölk, M.; Milkovska-Stamenova, S.; Hoffmann, R. Comprehensive profiling of the native and modified peptidomes of raw bovine milk and processed milk products. *Foods* **2020**, *9*, 1841. [CrossRef] [PubMed]
222. Zenker, H.E.; Van Lieshout, G.A.A.; Van Gool, M.P.; Bragt, M.C.E.; Hettinga, K.A. Lysine blockage of milk proteins in infant formula impairs overall protein digestibility and peptide release. *Food Funct.* **2020**, *11*, 358–369. [CrossRef]
223. Sarriá, B.; López-Fandiño, R.; Vaquero, M.P. Protein nutritive utilization in rats fed powder and liquid infant formulas/Utilización nutritiva de la proteína en ratas alimentadas con formulas infantiles en polvo y líquidas. *Food Sci. Technol. Int.* **2000**, *6*, 9–16. [CrossRef]
224. Contreras-Calderón, J.; Guerra-Hernández, E.; García-Villanova, B. Indicators of non-enzymatic browning in the evaluation of heat damage of ingredient proteins used in manufactured infant formulas. *Eur. Food Res. Technol.* **2008**, *227*, 117–124. [CrossRef]
225. Meltretter, J.; Birlouez-Aragon, I.; Becker, C.M.; Pischetsrieder, M. Assessment of heat treatment of dairy products by MALDI-TOF-MS. *Mol. Nutr. Food Res.* **2009**, *53*, 1487–1495. [CrossRef]
226. Wada, Y.; Lönnerdal, B. Effects of different industrial heating processes of milk on site-specific protein modifications and their relationship to in vitro and in vivo digestibility. *J. Agric. Food Chem.* **2014**, *62*, 4175–4185. [CrossRef]
227. Lee, H.M.; Yang, S.Y.; Han, J.; Kim, Y.K.; Kim, Y.J.; Rhee, M.S.; Lee, K.W. Optimization of spray drying parameters and food additives to reduce glycation using response surface methodology in powdered infant formulas. *Food Sci. Biotechnol.* **2019**, *28*, 769–777. [CrossRef]
228. Cattaneo, S.; Masotti, F.; Pellegrino, L. Liquid infant formulas: Technological tools for limiting heat damage. *J. Agric. Food Chem.* **2009**, *57*, 10689–10694. [CrossRef]

229. Oh, N.S.; Young Lee, J.; Lee, H.A.; Joung, J.Y.; Shin, Y.K.; Kim, S.H.; Kim, Y.; Lee, K.W. Chemical characteristics and enhanced hepatoprotective activities of Maillard reaction products derived from milk protein-sugar system. *J. Dairy Sci.* **2016**, *99*, 947–958. [CrossRef] [PubMed]
230. Li, Z.; Luo, Y.; Feng, L. Effects of Maillard reaction conditions on the antigenicity of α-lactalbumin and β-lactoglobulin in whey protein conjugated with maltose. *Eur. Food Res. Technol.* **2011**, *233*, 387–394. [CrossRef]
231. Heppell, L.M.J.; Cant, A.J.; Kilshaw, P.J. Reduction in the antigenicity of whey proteins by heat treatment: A possible strategy for producing a hypoallergenic infant milk formula. *Br. J. Nutr.* **1984**, *51*, 29–36. [CrossRef] [PubMed]
232. Kleber, N.; Krause, I.; Illgner, S.; Hinrichs, J. The antigenic response of β-lactoglobulin is modulated by thermally induced aggregation. *Eur. Food Res. Technol.* **2004**, *219*, 105–110. [CrossRef]
233. Ehn, B.M.; Ekstrand, B.; Bengtsson, U.; Ahlstedt, S. Modification of IgE Binding during Heat Processing of the Cow's Milk Allergen β-Lactoglobulin. *J. Agric. Food Chem.* **2004**, *52*, 1398–1403. [CrossRef]
234. Bu, G.; Luo, Y.; Zheng, Z.; Zheng, H. Effect of heat treatment on the antigenicity of bovine α-lactalbumin and β-lactoglobulin in whey protein isolate. *Food Agric. Immunol.* **2009**, *20*, 195–206. [CrossRef]
235. Xu, Q.; Shi, J.; Yao, M.; Jiang, M.; Luo, Y. Effects of heat treatment on the antigenicity of four milk proteins in milk protein concentrates. *Food Agric. Immunol.* **2016**, *27*, 401–413. [CrossRef]
236. Taheri-Kafrani, A.; Gaudin, J.C.; Rabesona, H.; Nioi, C.; Agarwal, D.; Drouet, M.; Chobert, J.M.; Bordbar, A.K.; Haertle, T. Effects of heating and glycation of β-lactoglobulin on its recognition by ige of sera from cow milk allergy patients. *J. Agric. Food Chem.* **2009**, *57*, 4974–4982. [CrossRef]
237. Roth-Walter, F.; Berin, M.C.; Arnaboldi, P.; Escalante, C.R.; Dahan, S.; Rauch, J.; Jensen-Jarolim, E.; Mayer, L. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy Eur. J. Allergy Clin. Immunol.* **2008**, *63*, 882–890. [CrossRef]
238. Graversen, K.B.; Ballegaard, A.S.R.; Kræmer, L.H.; Hornslet, S.E.; Sørensen, L.V.; Christoffersen, H.F.; Jacobsen, L.N.; Untersmayr, E.; Smit, J.J.; Bøgh, K.L. Cow's milk allergy prevention and treatment by heat-treated whey—A study in Brown Norway rats. *Clin. Exp. Allergy* **2020**, *50*, 708–721. [CrossRef]
239. Kleber, N.; Maier, S.; Hinrichs, J. Antigenic response of bovine β-lactoglobulin influenced by ultra-high pressure treatment and temperature. *Innov. Food Sci. Emerg. Technol.* **2007**, *8*, 39–45. [CrossRef]
240. Zhong, J.; Liu, C.; Liu, W.; Cai, X.; Tu, Z.; Wan, J. Effect of dynamic high-pressure microfluidization at different temperatures on the antigenic response of bovine β-lactoglobulin. *Eur. Food Res. Technol.* **2011**, *233*, 95–102. [CrossRef]
241. Chicón, R.; Belloque, J.; Alonso, E.; López-Fandiño, R. Immunoreactivity and digestibility of high-pressure-treated whey proteins. *Int. Dairy J.* **2008**, *18*, 367–376. [CrossRef]
242. Boughellout, H.; Choiset, Y.; Rabesona, H.; Chobert, J.M.; Haertle, T.; Mounir, S.; Allaf, K.; Zidoune, M.N. Effect of instant controlled pressure drop (DIC) treatment on milk protein's immunoreactivity. *Food Agric. Immunol.* **2015**, *26*, 71–81. [CrossRef]
243. Odueke, O.B.; Farag, K.W.; Baines, R.N.; Chadd, S.A. Irradiation Applications in Dairy Products: A Review. *Food Bioprocess Technol.* **2016**, *9*, 751–767. [CrossRef]
244. Tammineedi, C.V.R.K.; Choudhary, R.; Perez-Alvarado, G.C.; Watson, D.G. Determining the effect of UV-C, high intensity ultrasound and nonthermal atmospheric plasma treatments on reducing the allergenicity of α-casein and whey proteins. *LWT—Food Sci. Technol.* **2013**, *54*, 35–41. [CrossRef]
245. Hu, G.; Zheng, Y.; Liu, Z.; Deng, Y.; Zhao, Y. Structure and IgE-binding properties of α-casein treated by high hydrostatic pressure, UV-C, and far-IR radiations. *Food Chem.* **2016**, *204*, 46–55. [CrossRef]
246. Lee, J.W.; Kim, J.H.; Yook, H.S.; Kang, K.O.; Lee, S.Y.; Hwang, H.J.; Byun, M.W. Effects of gamma radiation on the allergenic and antigenic properties of milk proteins. *J. Food Prot.* **2001**, *64*, 272–276. [CrossRef]
247. Stanic-Vucinic, D.; Stojadinovic, M.; Atanaskovic-Markovic, M.; Ognjenovic, J.; Grönlund, H.; van Hage, M.; Lantto, R.; Sancho, A.I.; Velickovic, T.C. Structural changes and allergenic properties of β-lactoglobulin upon exposure to high-intensity ultrasound. *Mol. Nutr. Food Res.* **2012**, *56*, 1894–1905. [CrossRef]
248. Tammineedi, C.V.R.K.; Choudhary, R. Recent Advances in Processing for Reducing Dairy and Food Allergenicity. *Int. J. Food Sci. Nutr. Eng.* **2014**, *4*, 36–42.
249. Xiang, B.Y.; Ngadi, M.O.; Ochoa-Martinez, L.A.; Simpson, M.V. Pulsed Electric Field-Induced Structural Modification of Whey Protein Isolate. *Food Bioprocess Technol.* **2011**, *4*, 1341–1348. [CrossRef]
250. Meyer, R.; Groetch, M.; Venter, C. When Should Infants with Cow's Milk Protein Allergy Use an Amino Acid Formula? A Practical Guide. *J. Allergy Clin. Immunol. Pract.* **2017**, *6*, 383–399. [CrossRef] [PubMed]
251. Vanderhoof, J.A. In time: Misuse and overuse of amino acid formulas in cow milk allergy. *Rev. Paul. Pediatr.* **2015**, *33*, 379–380. [CrossRef] [PubMed]
252. Sicherer, S.H.; Noone, S.A.; Koerner, C.B.; Christie, L.; Burks, A.W.; Sampson, H.A. Hypoallergenicity and efficacy of an amino acid-based formula in children with cow's milk and multiple food hypersensitivities. *J. Pediatr.* **2001**, *138*, 688–693. [CrossRef]
253. Nowak-Wagrzyn, A.; Czerkies, L.A.; Collins, B.; Saavedra, J.M. Evaluation of hypoallergenicity of a new, amino acid-based formula. *Clin. Pediatr.* **2015**, *54*, 264–272. [CrossRef]
254. Canani, R.B.; Nocerino, R.; Frediani, T.; Lucarelli, S.; Di Scala, C.; Varin, E.; Leone, L.; Muraro, A.; Agostoni, C. Amino acid-based formula in cow's milk allergy: Long-term effects on body growth and protein metabolism. *J. Pediatr. Gastroenterol. Nutr.* **2017**, *64*, 632–638. [CrossRef]

255. Corkins, M.; Czerkies, L.A.; Storm, H.M.; Sun, S.; Saavedra, J.M. Assessment of Growth of Infants Fed an Amino Acid-Based Formula. *Clin. Med. Insights Pediatr.* **2016**, *10*, CMPed.S33071. [CrossRef]
256. Vandenplas, Y.; Dupont, C.; Eigenmann, P.; Heine, R.G.; Høst, A.; Järvi, A.; Kuitunen, M.; Mukherjee, R.; Ribes-Koninckx, C.; Szajewska, H.; et al. Growth in Infants with Cow's Milk Protein Allergy Fed an Amino AcidBased Formula. *Pediatr. Gastroenterol. Hepatol. Nutr.* **2021**, *24*, 392–402. [CrossRef]
257. Panel, E.; Nda, A. Scientific Opinion on the suitability of goat milk protein as a source of protein in infant formulae and in follow-on formulae. *EFSA J.* **2012**, *10*, 2603.
258. Faye, B.; Konuspayeva, G. The sustainability challenge to the dairy sector—The growing importance of non-cattle milk production worldwide. *Int. Dairy J.* **2012**, *24*, 50–56. [CrossRef]
259. Hackmann, T.J.; Spain, J.N. Invited review: Ruminant ecology and evolution: Perspectives useful to ruminant livestock research and production. *J. Dairy Sci.* **2010**, *93*, 1320–1334. [CrossRef] [PubMed]
260. Burger, P.A.; Ciani, E.; Faye, B. Old World camels in a modern world—A balancing act between conservation and genetic improvement. *Anim. Genet.* **2019**, *50*, 598–612. [CrossRef] [PubMed]
261. Alberto, F.J.; Boyer, F.; Orozco-Terwengel, P.; Streeter, I.; Servin, B.; De Villemereuil, P.; Benjelloun, B.; Librado, P.; Biscarini, F.; Colli, L.; et al. Convergent genomic signatures of domestication in sheep and goats. *Nat. Commun.* **2018**, *9*, 813. [CrossRef]
262. Brosnahan, M.M. Genetics, Evolution, and Physiology of Donkeys and Mules. *Vet. Clin. N. Am. Equine Pract.* **2019**, *35*, 457–467. [CrossRef]
263. Maryniak, N.Z.; Hansen, E.B.; Ballegaard, A.-S.R.; Sancho, A.I.; Bøgh, K.L. Comparison of the allergenicity and immunogenicity of Camel and cow's milk—A study in brown Norway rats. *Nutrients* **2018**, *10*, 1903. [CrossRef]
264. Ribeiro, A.C.; Ribeiro, S.D.A. Specialty products made from goat milk. *Small Rumin. Res.* **2010**, *89*, 226–234. [CrossRef]
265. Prosser, C.G. Compositional and functional characteristics of goat milk and relevance as a base for infant formula. *J. Food Sci.* **2021**, *86*, 257–265. [CrossRef]
266. Ceballos, L.S.; Morales, E.R.; de la Torre Adarve, G.; Castro, J.D.; Martínez, L.P.; Sampelayo, M.R.S. Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. *J. Food Compos. Anal.* **2009**, *22*, 322–329. [CrossRef]
267. Park, Y.W. Hypo-allergenic and therapeutic significance of goat milk. *Small Rumin. Res.* **1994**, *14*, 151–159. [CrossRef]
268. Restani, P. Goat milk allerginicity. *J. Pediatr. Gastroenterol. Nutr.* **2004**, *39*, 323–324. [CrossRef]
269. Jandal, J.M. Comparative aspects of goat and sheep milk. *Small Rumin. Res.* **1996**, *22*, 177–185. [CrossRef]
270. Bellioni-Businco, B.; Paganelli, R.; Lucenti, P.; Giampietro, P.G.; Perborn, H.; Businco, L. Allergenicity of goat's milk in children with cow's milk allergy. *J. Allergy Clin. Immunol.* **1999**, *103*, 1191–1194. [CrossRef]
271. Rodríguez del Río, P.; Sánchez-García, S.; Escudero, C.; Pastor-Vargas, C.; Sánchez Hernández, J.J.; Pérez-Rangel, I.; Ibáñez, M.D. Allergy to goat's and sheep's milk in a population of cow's milk-allergic children treated with oral immunotherapy. *Pediatr. Allergy Immunol.* **2012**, *23*, 128–132. [CrossRef]
272. Infante Pina, D.; Tormo Carnice, R.; Conde Zandueta, M. Use of goat's milk in patients with cow's milk allergy. *An. Pediatr.* **2003**, *59*, 138–142. [CrossRef]
273. Ah-Leung, S.; Bernard, H.; Bidat, E.; Paty, E.; Rancé, F.; Scheinmann, P.; Wal, J.M. Allergy to goat and sheep milk without allergy to cow's milk. *Allergy Eur. J. Allergy Clin. Immunol.* **2006**, *61*, 1358–1365. [CrossRef] [PubMed]
274. Caballer, H.; Gonz, R. Selective allergy to sheep's and goat's milk proteins. *Allergol. Immunopathol.* **2004**, *32*, 49–52.
275. Tavares, B.; Pereira, C.; Rodrigues, F.; Loureiro, G.; Chieira, C. Goat's milk allergy. *Allergol. Immunopathol.* **2007**, *35*, 113–116. [CrossRef]
276. Hazebrouck, S.; Ah-Leung, S.; Bidat, E.; Paty, E.; Drumare, M.F.; Tilleul, S.; Adel-Patient, K.; Wal, J.M.; Bernard, H. Goat's milk allergy without cow's milk allergy: Suppression of non-cross-reactive epitopes on caprine β-casein. *Clin. Exp. Allergy* **2013**, *44*, 602–610. [CrossRef]
277. Bernard, H.; Ah-Leung, S.; Tilleul, S.; Drumare, M.F.; Paty, E.; Bidat, E.; Wal, J.M.; Hazebrouck, S. Specificity of IgE antibodies from patients allergic to goat's milk and tolerant to cow's milk determined with plasmin-derived peptides of bovine and caprine β-caseins. *Mol. Nutr. Food Res.* **2012**, *56*, 1532–1540. [CrossRef]
278. Lara-Villoslada, F.; Olivares, M.; Jiménez, J.; Boza, J.; Xaus, J. Goat milk is less immunogenic than cow milk in a murine model of atopy. *J. Pediatr. Gastroenterol. Nutr.* **2004**, *39*, 354–360. [CrossRef]
279. Ceballos, L.S.; Sampelayo, M.R.S.; Extremera, F.G.; Osorio, M.R. Evaluation of the allergenicity of goat milk, cow milk, and their lactosera in a guinea pig model. *J. Dairy Sci.* **2009**, *92*, 837–846. [CrossRef] [PubMed]
280. Mohapatra, A.; Shinde, A.K.; Singh, R. Sheep milk: A pertinent functional food. *Small Rumin. Res.* **2019**, *181*, 6–11. [CrossRef]
281. Balthazar, C.F.; Pimentel, T.C.; Ferrão, L.L.; Almada, C.N.; Santillo, A.; Albenzio, M.; Mollakhalili, N.; Mortazavian, A.M.; Nascimento, J.S.; Silva, M.C.; et al. Sheep Milk: Physicochemical Characteristics and Relevance for Functional Food Development. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 247–262. [CrossRef] [PubMed]
282. Ministry for Primary Industries. *Labelling of Retail Ready Infant Formula and Formulated Sypplementary Food for Young Children*; Ministry for Primary Industries: Wellington, New Zealand, 2018.
283. Jianshui County Honghui Aquaculture Industry. Baby Formula Milk Powder Containing Desalted Sheep Whey Powder and Production Method of Baby Formula Milk Powder. Patent Application No.CN106615147A, 10 May 2017.
284. Calvani, M.; Alessandri, C. Anaphylaxis to sheep's milk cheese in a child unaffected by cow's milk protein allergy. *Eur. J. Pediatr.* **1998**, *157*, 17–19. [CrossRef]

285. Wüthrich, B.; Johansson, S.G.O. Allergy to cheese produced from sheep's and goat's milk but not to cheese produced from cow's milk. *J. Allergy Clin. Immunol.* **1995**, *96*, 270–273. [CrossRef]
286. Viñas, M.; Carnés, J.; López-Matas, M.A.; Hernández, N.; Castillo, M.J.; Ibero, M. Allergy to goat and sheep cheese with tolerance to cow's milk and its derivatives. *Allergol. Immunopathol.* **2014**, *42*, 186–190. [CrossRef]
287. Umpiérrez, A.; Quirce, S.; Marañón, F.; Cuesta, J.; García-Villamuza, Y.; Lahoz, C.; Sastre, J. Allergy to goat and sheep cheese with good tolerance to cow cheese. *Clin. Exp. Allergy* **1999**, *29*, 1064–1068. [CrossRef]
288. Pazheri, F.; Melton, A.L.; Poptic, E.; Wallard, B. Allergy to Sheep Milk With Or Without Allergy To Cow Milk. *J. Allergy Clin. Immunol.* **2014**, *133*, AB199. [CrossRef]
289. Barlowska, J.; Szwajkowska, M.; Litwińczuk, Z.; Król, J. Nutritional Value and Technological Suitability of Milk from Various Animal Species Used for Dairy Production. *Compr. Rev. Food Sci. Food Saf.* **2011**, *10*, 291–302. [CrossRef]
290. Vincenzetti, S.; Cammertoni, N.; Rapaccetti, R.; Santini, G.; Klimanova, Y.; Zhang, J.-J.; Polidori, P. Nutraceutical and Functional Properties of Camelids' Milk. *Beverages* **2022**, *8*, 12. [CrossRef]
291. Hailu, Y.; Hansen, E.B.; Seifu, E.; Eshetu, M.; Ipsen, R.; Kappeler, S. Functional and technological properties of camel milk proteins: A review. *J. Dairy Res.* **2016**, *83*, 422–429. [CrossRef] [PubMed]
292. Sélo, I.; Clément, G.; Bernard, H.; Chatel, J.M.; Créminon, C.; Peltre, G.; Wal, J.M. Allergy to bovine β-lactoglobulin: Specificity of human IgE to tryptic peptides. *Clin. Exp. Allergy* **1999**, *29*, 1055–1063. [CrossRef] [PubMed]
293. Omar, A.; Harbourne, N.; Oruna-Concha, M.J. Quantification of major camel milk proteins by capillary electrophoresis. *Int. Dairy J.* **2016**, *58*, 31–35. [CrossRef]
294. Swelum, A.A.; El-Saadony, M.T.; Abdo, M.; Ombarak, R.A.; Hussein, E.O.S.; Suliman, G.; Alhimaidi, A.R.; Ammari, A.A.; Ba-Awadh, H.; Taha, A.E.; et al. Nutritional, antimicrobial and medicinal properties of Camel's milk: A review. *Saudi J. Biol. Sci.* **2021**, *28*, 3126–3136. [CrossRef]
295. Berhe, T.; Seifu, E.; Ipsen, R.; Kurtu, M.Y.; Hansen, E.B. Processing Challenges and Opportunities of Camel Dairy Products. *Int. J. Food Sci.* **2017**, *2017*, 9061757. [CrossRef]
296. Fiocchi, A.; Bognanni, A.; Brożek, J.; Ebisawa, M.; Schünemann, H.; Ansotegui, I.J.; Arasi, S.; Assa'ad, A.H.; Bahna, S.L.; Canani, R.B.; et al. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines update—I—Plan and definitions. *World Allergy Organ. J.* **2022**, *15*, 100609. [CrossRef]
297. Navarrete-Rodríguez, E.M.; Ríos-Villalobos, L.A.; Alcocer-Arreguín, C.R.; Del-Rio-Navarro, B.E.; Del Rio-Chivardi, J.M.; Saucedo-Ramírez, O.J.; Sienra-Monge, J.J.L.; Frias, R.V. Cross-over clinical trial for evaluating the safety of camel's milk intake in patients who are allergic to cow's milk protein. *Allergol. Immunopathol.* **2017**, *46*, 149–154. [CrossRef]
298. Ehlayel, M.S.; Hazeima, K.A.; Al-Mesaifri, F.; Bener, A. Camel milk: An alternative for cow's milk allergy in children. *Allergy Asthma Proc.* **2011**, *32*, 255–258. [CrossRef]
299. Ehlayel, M.; Bener, A.; Abu Hazeima, K.; Al-Mesaifri, F. 1 Camel Milk Is a Safer Choice than Goat Milk for Feeding Children with Cow Milk Allergy. *ISRN Allergy* **2011**, *2011*, 391641. [CrossRef]
300. Aburiziza, A.J. Cross reactivity between cow's and camel's milk in the infant population of Saudi Arabia. *Curr. Pediatr. Res.* **2020**, *24*, 175–179.
301. Restani, P.; Beretta, B.; Fiocchi, A.; Ballabio, C.; Galli, C.L. Cross-reactivity between mammalian proteins. *Ann. Allergy Asthma Immunol.* **2002**, *89*, 11–15. [CrossRef]
302. Restani, P.; Gaiaschi, A.; Plebani, A.; Beretta, B.; Cagavni, G.; Fiocchi, A.; Poiesi, C.; Velona, T.; Ugazio, A.G.; Galli, C.L. Cross-reactivity between milk proteins from different animal species. *Clin. Exp. Allergy* **1999**, *29*, 997–1004. [CrossRef] [PubMed]
303. El-Agamy, E.I.; Nawar, M.; Shamsia, S.M.; Awad, S.; Haenlein, G.F.W. Are camel milk proteins convenient to the nutrition of cow milk allergic children? *Small Rumin. Res.* **2009**, *82*, 1–6. [CrossRef]
304. Vojdani, A.; Turnpaugh, C.; Vojdani, E. Immune reactivity against a variety of mammalian milks and plant-based milk substitutes. *J. Dairy Res.* **2018**, *85*, 358–365. [CrossRef] [PubMed]
305. Al-Hammadi, S.; El-Hassan, T.; Al-Reyami, L. Anaphylaxis to camel milk in an atopic child. *Allergy* **2010**, *65*, 1622–1623. [CrossRef]
306. Aspri, M.; Economou, N.; Papademas, P. Donkey milk: An overview on functionality, technology, and future prospects. *Food Rev. Int.* **2017**, *33*, 316–333. [CrossRef]
307. Polidori, P.; Beghelli, D.; Mariani, P.; Vincenzetti, S. Donkey milk production: State of the art. *Ital. J. Anim. Sci.* **2009**, *8*, 677–683. [CrossRef]
308. Martini, M.; Altomonte, I.; Licitra, R.; Salari, F. Nutritional and Nutraceutical Quality of Donkey Milk. *J. Equine Vet. Sci.* **2018**, *65*, 33–37. [CrossRef]
309. Sarti, L.; Martini, M.; Brajon, G.; Barni, S.; Salari, F.; Altomonte, I.; Ragona, G.; Mori, F.; Pucci, N.; Muscas, G.; et al. Donkey's Milk in the Management of Children with Cow's Milk protein allergy: Nutritional and hygienic aspects. *Ital. J. Pediatr.* **2019**, *45*, 102. [CrossRef]
310. Tesse, R.; Paglialunga, C.; Braccio, S.; Armenio, L. Adequacy and tolerance to ass's milk in an Italian cohort of children with cow's milk allergy. *Ital. J. Pediatr.* **2009**, *35*, 4–7. [CrossRef]
311. Barni, S.; Sarti, L.; Mori, F.; Muscas, G.; Belli, F.; Pucci, N.; Novembre, E. Tolerability and palatability of donkey's milk in children with cow's milk allergy. *Pediatr. Allergy Immunol.* **2018**, *29*, 329–331. [CrossRef] [PubMed]

312. Monti, G.; Bertino, E.; Muratore Cristina, M.; Coscia, A.; Cresi, F.; Silvestro, L.; Fabris, C.; Fortunato, D.; Giuffrida Gabriella, M.; Conti, A. Efficacy of donkey's milk in treating highly problematic cow's milk allergic children: An in vivo and in vitro study. *Pediatr. Allergy Immunol.* **2007**, *18*, 258–264. [CrossRef] [PubMed]
313. Monti, G.; Viola, S.; Baro, C.; Cresi, F.; Tovo, P.A.; Moro, G.; Ferrero, M.P.; Conti, A.; Bertino, E. Tolerability of donkey's milk in 92 highly-problematic cow's milk allergic children. *J. Biol. Regul. Homeost. Agents* **2012**, *26*, 75–82. [PubMed]
314. Vita, D.; Passalacqua, G.; Di Pasquale, G.; Caminiti, L.; Crisafulli, G.; Rulli, I.; Pajno, G.B. Ass's milk in children with atopic dermatitis and cow's milk allergy: Crossover comparison with goat's milk. *Pediatr. Allergy Immunol.* **2007**, *18*, 594–598. [CrossRef] [PubMed]
315. Martini, M.; Swiontek, K.; Antonicelli, L.; Garritani, M.S.; Bilò, M.B.; Mistrello, G.; Amato, S.; Revets, D.; Ollert, M.; Morisset, M.; et al. Lysozyme, a new allergen in donkey's milk. *Clin. Exp. Allergy* **2018**, *48*, 1521–1523. [CrossRef] [PubMed]
316. Peeters, C.; Herman, A.; Baeck, M. Donkey's milk allergy. *Br. J. Dermatol.* **2017**, *177*, 1760–1761. [CrossRef]
317. Giorgis, V.; Rolla, G.; Raie, A.; Geuna, M.; Boita, M.; Lamberti, C.; Nebbia, S.; Giribaldi, M.; Giuffrida, M.G.; Brussino, L.; et al. A case of work-related donkey milk allergy. *J. Investig. Allergol. Clin. Immunol.* **2018**, *28*, 197–199. [CrossRef]
318. Uniacke-Lowe, T.; Huppertz, T.; Fox, P.F. Equine milk proteins: Chemistry, structure and nutritional significance. *Int. Dairy J.* **2010**, *20*, 609–629. [CrossRef]
319. Doreau, M.; Martin-Rosset, W. Horse. In *Animals that Produce Dairy Foods*; Academic Press: Cambridge, MA, USA, 2011; pp. 358–364.
320. Businco, L.; Giampietro, P.G.; Lucenti, P.; Lucaroni, F.; Pini, C.; Di Felice, G.; Lacovacci, P.; Curadi, C.; Orlandi, M. Allergenicity of mare's milk in children with cow's milk allergy. *J. Allergy Clin. Immunol.* **2000**, *105*, 1031–1034. [CrossRef]
321. Fotschki, J.; Szyc, A.M.; Laparra, J.M.; Markiewicz, L.H.; Wróblewska, B. Immune-modulating properties of horse milk administered to mice sensitized to cow milk. *J. Dairy Sci.* **2016**, *99*, 9395–9404. [CrossRef]
322. Duan, C.; Ma, L.; Cai, L.; Li, X.; Ma, F.; Chen, J.; Huo, G.; Li, D. Comparison of allergenicity among cow, goat, and horse milks using a murine model of atopy. *Food Funct.* **2021**, *12*, 5417–5428. [CrossRef] [PubMed]
323. Verhulst, L.; Kerre, S.; Goossens, A. The unsuspected power of mare's milk. *Contact Dermat.* **2016**, *74*, 376–377. [CrossRef] [PubMed]
324. Fanta, C.; Ebner, C. Allergy to Mare's milk. *Allergy* **1998**, *53*, 539–540. [CrossRef] [PubMed]
325. Gall, H.; Kalveram, M.; Sick, H. Allergy to the heat-labile proteins alpha-lactalbumin and beta-lactoglobulin in mare's milk. *J. Allergy Clin. Immunol.* **1996**, *97*, 1304–1307. [CrossRef]
326. Fjeldsgaard, B.E.; Paulsen, B.S. Comparison of IgE-binding antigens in horse dander and a mixture of horse hair and skin scrapings. *Allergy* **1993**, *48*, 535–541. [CrossRef]
327. Heller, M.C.; Keoleian, G.A.; Willett, W.C. Toward a life cycle-based, diet-level framework for food environmental impact and nutritional quality assessment: A critical review. *Environ. Sci. Technol.* **2013**, *47*, 12632–12647. [CrossRef]
328. Martini, D.; Tucci, M.; Bradfield, J.; Di Giorgio, A.; Marino, M.; Del Bo', C.; Porrini, M.; Riso, P. Principles of sustainable healthy diets in worldwide dietary guidelines: Efforts so far and future perspectives. *Nutrients* **2021**, *13*, 1827. [CrossRef]
329. Green, H.; Broun, P.; Cook, D.; Cooper, K.; Drewnowski, A.; Pollard, D.; Sweeney, G.; Roulin, A. Healthy and sustainable diets for future generations. *J. Sci. Food Agric.* **2018**, *98*, 3219–3224. [CrossRef]
330. Craig, W.J.; Fresán, U. International analysis of the nutritional content and a review of health benefits of non-dairy plant-based beverages. *Nutrients* **2021**, *13*, 842. [CrossRef]
331. Mäkinen, O.E.; Wanhalinna, V.; Zannini, E.; Arendt, E.K. Foods for Special Dietary Needs: Non-dairy Plant-based Milk Substitutes and Fermented Dairy-type Products. *Crit. Rev. Food Sci. Nutr.* **2016**, *56*, 339–349. [CrossRef]
332. Bridges, M. Moo-ove over, cow's milk: The rise of plant-based dairy alternatives. *Pract. Gastroenterol.* **2018**, *42*, 20–27.
333. Fructuoso, I.; Romão, B.; Han, H.; Raposo, A.; Ariza-Montes, A.; Araya-Castillo, L.; Zandonadi, R.P. An overview on nutritional aspects of plant-based beverages used as substitutes for cow's milk. *Nutrients* **2021**, *13*, 2650. [CrossRef] [PubMed]
334. Sethi, S.; Tyagi, S.K.; Anurag, R.K. Plant-based milk alternatives an emerging segment of functional beverages: A review. *J. Food Sci. Technol.* **2016**, *53*, 3408–3423. [CrossRef] [PubMed]
335. Ziarno, M.; Cichońska, P. Lactic Acid Bacteria-Fermentable Cereal- and Pseudocereal- Based Beverages. *Microorganisms* **2021**, *9*, 2532. [CrossRef] [PubMed]
336. Merritt, R.J.; Fleet, S.E.; Fifi, A.; Jump, C.; Schwartz, S.; Sentongo, T.; Duro, D.; Rudolph, J.; Turner, J. North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition Position Paper: Plant-based Milks. *J. Pediatr. Gastroenterol. Nutr.* **2020**, *71*, 276–281. [CrossRef]
337. Le Louer, B.; Lemale, J.; Garcette, K.; Orzechowski, C.; Chalvon, A.; Girardet, J.P.; Tounian, P. Severe nutritional deficiences in young infants with inappropriate plant milk consumption. *Arch. Pediatr.* **2014**, *21*, 483–488. [CrossRef]
338. Vitoria Miñana, I. The nutritional limitations of plant-based beverages in infancy and childhood. *Nutr. Hosp.* **2017**, *34*, 1205–1214. [CrossRef]
339. Agostoni, C.; Axelsson, I.; Goulet, O.; Koletzko, B.; Michaelsen, K.F.; Puntis, J.; Rieu, D.; Rigo, J.; Shamir, R.; Szajewska, H.; et al. Soy protein infant formulae and follow-on formulae: A commentary by the ESPGHAN committee on nutrition. *J. Pediatr. Gastroenterol. Nutr.* **2006**, *42*, 352–361. [CrossRef]
340. Verduci, E.; Di Profio, E.; Cerrato, L.; Nuzzi, G.; Riva, L.; Vizzari, G.; D'Auria, E.; Giannì, M.L.; Zuccotti, G.; Peroni, D.G. Use of Soy-Based Formulas and Cow's Milk Allergy: Lights and Shadows. *Front. Pediatr.* **2020**, *8*, 736. [CrossRef]

341. Global Soy-Based Infant Formula Market Growth, Share, Size, Trends and Forecast (2021-2027). Report ID: Rn743009364. 2021. Available online: https://www.expertmarketresearch.com/reports/soy-based-infant-formula-market (accessed on 15 November 2021).
342. Vandenplas, Y.; De Greef, E.; Devreker, T.; Hauser, B. Soy infant formula: Is it that bad? *Acta Paediatr. Int. J. Paediatr.* **2011**, *100*, 162–166. [CrossRef]
343. Vandenplas, Y.; Castrellon, P.G.; Rivas, R.; Gutiérrez, C.J.; Garcia, L.D.; Jimenez, J.E.; Anzo, A.; Hegar, B.; Alarcon, P. Safety of soya-based infant formulas in children. *Br. J. Nutr.* **2014**, *111*, 1340–1360. [CrossRef] [PubMed]
344. Westmark, C.J. Soy-Based Therapeutic Baby Formulas: Testable Hypotheses Regarding the Pros and Cons. *Front. Nutr.* **2017**, *3*, 59. [CrossRef] [PubMed]
345. Katz, Y.; Gutierrez-Castrellon, P.; González, M.G.; Rivas, R.; Lee, B.W.; Alarcon, P. A comprehensive review of sensitization and allergy to soy-based products. *Clin. Rev. Allergy Immunol.* **2014**, *46*, 272–281. [CrossRef] [PubMed]
346. Kattan, J.D.; Cocco, R.R.; Järvinen, K.M. Milk and Soy Allergy. *Pediatr. Clin. N. Am.* **2011**, *58*, 407–426. [CrossRef]
347. Kerner, J. Use of Infant Formulas in Preventing or Postponing Atopic Manifestations. *J. Pediatr. Gastroenterol. Nutr.* **1997**, *24*, 442–446. [CrossRef]
348. Rozenfeld, P.; Docena, G.H.; Añón, M.C.; Fossati, C.A. Detection and identification of a soy protein component that cross-reacts with caseins from cow's milk. *Clin. Exp. Immunol.* **2002**, *130*, 49–58. [CrossRef]
349. Giampietro, P.G.; Ragno, V.; Daniele, S.; Cantani, A.; Ferrara, M.; Businco, L. Soy hypersensitivity in children with food allergy. *Ann. Allergy* **1992**, *69*, 143–146.
350. Bock, S.A.; Atkins, F.M. Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges. *J. Pediatr.* **1990**, *117*, 561–567. [CrossRef]
351. Klemola, T.; Vanto, T.; Juntunen-Backman, K.; Kalimo, K.; Korpela, R.; Varjonen, E. Allergy to soy formula and to extensively hydrolyzed whey formula in infants with cow's milk allergy: A prospective, randomized study with a follow-up to the age of 2 years. *J. Pediatr.* **2002**, *140*, 219–224. [CrossRef]
352. Zeiger, R.S.; Sampson, H.A.; Bock, S.A.; Burks, A.W.; Harden, K.; Noone, S.; Martin, D.; Leung, S.; Wilson, G. Soy allergy in infants and children with IgE-associated cow's milk allergy. *J. Pediatr.* **1999**, *134*, 614–622. [CrossRef]
353. Johnstone, D.; Dutton, A. Dietary Prophylaxis of Allergic Disease in Children. *N. Engl. J. Med.* **1994**, *274*, 715–719. [CrossRef] [PubMed]
354. Glaster, J. Prophylaxis of Allergic Disease in the Newborn. *J. Am. Med. Assoc.* **1953**, *153*, 620–622. [CrossRef] [PubMed]
355. Kjellman, N.I.; Johansson, S.G. Soy versus cow's milk in infants with a biparental history of atopic disease: Development of atopic disease and immunoglobulins from birth to 4 years of age. *Clin. Allergy* **1979**, *9*, 347–358. [CrossRef] [PubMed]
356. Gruskay, F.L. Comparison of Breast, Cow, and Soy Feedings in the Prevention of Onset of Allergic Disease: A 15-Year Prospective Study. *Clin. Pediatr.* **1982**, *21*, 486–491. [CrossRef]
357. Osborn, D.A.; Sinn, J.K. Soy formula for prevention of allergy and food intolerance in infants. *Cochrane Database Syst Rev.* **2004**, *3*, CD003741.
358. Vale, S.L.; Lobb, M.; Netting, M.J.; Murray, K.; Clifford, R.; Campbell, D.E.; Salter, S.M. A systematic review of infant feeding food allergy prevention guidelines—Can we AGREE? *World Allergy Organ. J.* **2021**, *14*, 100550. [CrossRef]
359. Terracciano, L.; Bouygue, G.R.; Sarratud, T.; Veglia, F.; Martelli, A.; Fiocchi, A. Impact of dietary regimen on the duration of cow's milk allergy: A random allocation study. *Clin. Exp. Allergy* **2010**, *40*, 637–642. [CrossRef]
360. Berni Canani, R.; Nocerino, R.; Terrin, G.; Frediani, T.; Lucarelli, S.; Cosenza, L.; Passariello, A.; Leone, L.; Granata, V.; Di Costanzo, M.; et al. Formula selection for management of children with cow's milk allergy influences the rate of acquisition of tolerance: A prospective multicenter study. *J. Pediatr.* **2013**, *163*, 771–777.e1. [CrossRef]
361. Juliano, B.O.; Tuaño, A.P.P. Gross structure and composition of the rice grain. In *Rice: Chemistry and Technology*; Woodhead Publishing: Sawston, UK, 2018; pp. 31–53.
362. Amagliani, L.; O'Regan, J.; Kelly, A.L.; O'Mahony, J.A. Composition and protein profile analysis of rice protein ingredients. *J. Food Compos. Anal.* **2017**, *59*, 18–26. [CrossRef]
363. Amagliani, L.; O'Regan, J.; Kelly, A.L.; O'Mahony, J.A. The composition, extraction, functionality and applications of rice proteins: A review. *Trends Food Sci. Technol.* **2017**, *64*, 1–12. [CrossRef]
364. Dupont, C.; Bocquet, A.; Tomé, D.; Bernard, M.; Campeotto, F.; Dumond, P.; Essex, A.; Frelut, M.L.; Guénard-Bilbault, L.; Lack, G.; et al. Hydrolyzed rice protein-based formulas, a vegetal alternative in cow's milk allergy. *Nutrients* **2020**, *12*, 2654. [CrossRef] [PubMed]
365. Bocquet, A.; Dupont, C.; Chouraqui, J.P.; Darmaun, D.; Feillet, F.; Frelut, M.L.; Girardet, J.P.; Hankard, R.; Lapillonne, A.; Rozé, J.C.; et al. Efficacy and safety of hydrolyzed rice-protein formulas for the treatment of cow's milk protein allergy. *Arch. Pediatr.* **2019**, *26*, 238–246. [CrossRef] [PubMed]
366. Communities TC of the E. Comission Directive 1999/21/EC of 25 March 1999 on Dietary Foods for Special Medical Purposes; pp. 1–11. 1999, 79. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1999L0021:20070119:EN:PDF (accessed on 15 January 2022).
367. Regulation (EU) No. 609/2013 of the European Parlament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/. *Off. J. Eur. Union*. 2013. Available online: https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX:32013R0609 (accessed on 15 January 2022).

368. Agostoni, C.; Fiocchi, A.; Riva, E.; Terracciano, L.; Sarratud, T.; Martelli, A.; Lodi, F.; D'Auria, E.; Zuccotti, G.; Giovannini, M. Growth of infants with IgE-mediated cow's milk allergy fed different formulas in the complementary feeding period. *Pediatr. Allergy Immunol.* **2007**, *18*, 599–606. [CrossRef]
369. Vandenplas, Y.; De Greef, E.; Hauser, B. Safety and tolerance of a new extensively hydrolyzed rice protein-based formula in the management of infants with cow's milk protein allergy. *Eur. J. Pediatr.* **2014**, *173*, 1209–1216. [CrossRef]
370. Fiocchi, A.; Travaini, M.; D'Auria, E.; Banderali, G.; Bernardo, L.; Riva, E. Tolerance to a rice hydrolysate formula in children allergic to cow's milk and soy. *Clin. Exp. Allergy* **2003**, *33*, 1576–1580. [CrossRef]
371. Tzifi, F.; Grammeniatis, V.; Papadopoulos, M. Soy- and rice-based formula and infant allergic to cow's milk. *Endocr. Metab. Immune Disord.-Drug Targets* **2014**, *14*, 38–46. [CrossRef]
372. Fiocchi, A.; Restani, P.; Bernardini, R.; Lucarelli, S.; Lombardi, G.; Magazzù, G.; Marseglia, G.L.; Pittschieler, K.; Tripodi, S.; Troncone, R.; et al. A hydrolysed rice-based formula is tolerated by children with cow's milk allergy: A multi-centre study. *Clin. Exp. Allergy* **2006**, *36*, 311–316. [CrossRef]
373. Reche, M.; Pascual, C.; Fiandor, A.; Polanco, I.; Rivero-Urgell, M.; Chifre, R.; Johnston, S.; Martín-Esteban, M. The effect of a partially hydrolysed formula based on rice protein in the treatment of infants with cow's milk protein allergy. *Pediatr. Allergy Immunol.* **2010**, *21*, 577–585. [CrossRef]
374. Venlet, N.V.; Hettinga, K.A.; Schebesta, H.; Bernaz, N. Perspective: A Legal and Nutritional Perspective on the Introduction of Quinoa-Based Infant and Follow-on Formula in the EU. *Adv. Nutr.* **2021**, *12*, 1100–1107. [CrossRef]
375. Le Roux, L.; Ménard, O.; Chacon, R.; Dupont, D.; Jeantet, R.; Deglaire, A.; Nau, F. Are faba bean and pea proteins potential whey protein substitutes in infant formulas? An in vitro dynamic digestion approach. *Foods* **2020**, *9*, 362. [CrossRef] [PubMed]
376. Le Roux, L.; Chacon, R.; Dupont, D.; Jeantet, R.; Deglaire, A.; Nau, F. In vitro static digestion reveals how plant proteins modulate model infant formula digestibility. *Food Res. Int.* **2020**, *130*, 108917. [CrossRef] [PubMed]
377. Le Roux, L.; Mejean, S.; Chacon, R.; Lopez, C.; Dupont, D.; Deglaire, A.; Nau, F.; Jeantet, R. Plant proteins partially replacing dairy proteins greatly influence infant formula functionalities. *LWT* **2020**, *120*, 108891. [CrossRef]
378. Alonso-Miravalles, L.; Barone, G.; Waldron, D.; Bez, J.; Joehnke, M.S.; Petersen, I.L.; Zannini, E.; Arendt, E.K.; O'Mahony, J.A. Formulation, pilot-scale preparation, physicochemical characterization and digestibility of a lentil protein-based model infant formula powder. *J. Sci. Food Agric.* **2021**, 1–11. [CrossRef] [PubMed]
379. Schuh, S.; Thevenier, A.; Ran-Ressler, R.; Rade-Kukic, K.; Kuslys, M. Infant Formula for Cow's Milk Protein Allergic Infants. U.S. Patent Application 16/332,181, 29 August 2019.
380. Ulloa, J.A.; Valencia, M.E.; Garcia, Z.H. Protein Concentrate from Chickpea: Nutritive Value of a Protein Concentrate from Chickpea (*Cicer arietinum*) Obtained by Ultrafiltration and Its Potential Use in an Infant Formula. *J. Food Sci.* **1988**, *53*, 1396–1398. [CrossRef]
381. Malunga, L.N.; Bar-El Dadon, S.; Zinal, E.; Berkovich, Z.; Abbo, S.; Reifen, R. The potential use of chickpeas in development of infant follow-on formula. *Nutr. J.* **2014**, *13*, 8. [CrossRef] [PubMed]
382. Turck, D.; Bresson, J.L.; Burlingame, B.; Dean, T.; Fairweather-Tait, S.; Heinonen, M.; Hirsch-Ernst, K.I.; Mangelsdorf, I.; McArdle, H.; Naska, A.; et al. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. *EFSA J.* **2016**, *14*, e04594.
383. Floris, R.; Lambers, T.; Alting, A.; Kiers, J. Trends in infant formulas: A dairy perspective. In *Improving the Safety and Quality of Milk: Improving Quality in Milk Products*; Woodhead Publishing Limited: Sawston, UK, 2010; pp. 454–474. [CrossRef]
384. Hildebrand, H.V.; Arias, A.; Simons, E.; Gerdts, J.; Povolo, B.; Rothney, J.; Protudjer, J.L.P. Adult and Pediatric Food Allergy to Chickpea, Pea, Lentil, and Lupine: A Scoping Review. *J. Allergy Clin. Immunol. Pract.* **2021**, *9*, 290–301. [CrossRef]
385. Verma, A.K.; Kumar, S.; Das, M.; Dwivedi, P.D. A comprehensive review of legume allergy. *Clin. Rev. Allergy Immunol.* **2013**, *45*, 30–46. [CrossRef]
386. Soller, L.; La Vieille, S.; Cameron, S.B.; Mak, R.; Cook, V.E.; Gerdts, J.; Chan, E.S. Allergic reactions to emerging food allergens in Canadian children. *Allergy Asthma Clin. Immunol.* **2021**, *17*, 71. [CrossRef]
387. Damiani, E.; Aloia, A.M.; Priore, M.G.; Pastore, A.; Nardulli, S.; Lippolis, C.; Macchia, L.; Ferrannini, A. Vicia faba Hypersensitivity and ASA Intolerance in a Farmer: A Case Report. *J. Allergy* **2011**, *2011*, 191787. [CrossRef] [PubMed]
388. Kumar, D.; Kumar, S.; Verma, A.K.; Sharma, A.; Tripathi, A.; Chaudhari, B.P.; Kant, S.; Das, M.; Jain, S.K.; Dwivedi, P.D. Hypersensitivity linked to exposure of broad bean protein(s) in allergic patients and BALB/c mice. *Nutrition* **2014**, *30*, 903–914. [CrossRef] [PubMed]
389. Taylor, S.L.; Marsh, J.T.; Koppelman, S.J.; Kabourek, J.L.; Johnson, P.E.; Baumert, J.L. A perspective on pea allergy and pea allergens. *Trends Food Sci. Technol.* **2021**, *116*, 186–198. [CrossRef]
390. Ruales, J.; de Grijalva, Y.; Lopez-Jaramillo, P.; Nair, B.M. The nutritional quality of an infant food from quinoa and its effect on the plasma level of insulin-like growth factor-1 (IGF-1) in undernourished children. *Int. J. Food Sci. Nutr.* **2002**, *53*, 143–154. [CrossRef] [PubMed]
391. Rojas, W.; Alandia, G.; Irigoyen, J.; Blajos, J.; Santivañez, T. *Quinoa: An Ancient Crop to Contribute to World Food Security*; FAO Regional Office for Latin America and the Caribbean: Santiago, Chile, 2011; Volume 37, p. 66.
392. Ballegaard, A.S.R.; Larsen, J.M.; Rasmussen, P.H.; Untersmayr, E.; Pilegaard, K.; Bøgh, K.L. Quinoa (Chenopodium quinoa Willd.) Seeds Increase Intestinal Protein Uptake. *Mol. Nutr. Food Res.* **2021**, *65*, 2100102. [CrossRef]

393. Astier, C.; Moneret-Vautrin, D.A.; Puillandre, E.; Bihain, B. First case report of anaphylaxis to quinoa, a novel food in France. *Allergy* **2008**, *64*, 818–819. [CrossRef]
394. Guarnieri, G.; Bonadonna, P.; Olivieri, E.; Schiappoli, M. Occupational asthma induced by exposure to Quinoa. *J. Investig. Allergol. Clin. Immunol.* **2019**, *29*, 451–452. [CrossRef]
395. Hong, J.; Convers, K.; Reeves, N.; Temparo, J. Anaphylaxis to quinoa. *Ann. Allergy Asthma Immunol.* **2013**, *10*, 60–61. [CrossRef]
396. Ballegaard, A.S.R.; Sancho, A.I.; Knudsen, N.H.; Gupta, S.; Pilegaard, K.; Bøgh, K.L. Quinoa allergenicity—Identification of sensitising and cross-reactive quinoa proteins. *Allergy Eur. J. Allergy Clin. Immunol.* **2021**, *76*, 338–339.
397. Kobayashi, T.; Nakamura, M.; Matsunaga, K.; Nakata, J.; Tagami, K.; Sato, N.; Kawabe, T.; Kondo, Y. Anaphylaxis due to potato starch (possibly caused by percutaneous sensitization). *Asia Pac. Allergy* **2021**, *11*, 5–9. [CrossRef]

2 Background

2.1 Alternative infant formulas

Population growth and climate change are one of the main factors for an increased focus on new, alternative protein sources for human feeding. This leads to an increased development of plant-based diets that could be reflected in an increased number of plant-based products available on the market [1,2]. For the dairy industry sector, the consumption of non-cattle milks has increased for the last years [3], which can be reflected in the availability of dairy products based on goat, sheep, horse, donkey and camel milk, depending on the region of the world [4].

From the perspective of infants feeding, at present, cow's milk is the most common source of proteins used in the infant formula production, yet it is also the most common cause of food allergy in infants and small children [5]. Therefore, for cow's milk allergy (CMA) management when breastfeeding is not possible, use of hypoallergenic infant formula based on hydrolysed cow's milk proteins is recommended [6].

Proteins from the non-cattle milks are gaining an interest for their utility in the production of alternative infant formulas for CMA management and prevention. Non-cattle and non-bovine milk proteins from horse, donkey and camel due to their proteins low sequence identity with cow's milk proteins, have a potency for their low cross-reactivity [7]. On the other hand, goat and sheep milk, because they are non-cattle but bovine milks, thus they are more closely related to cow's, have a high sequence identity with cow's milk proteins [7], and several studies showed their high cross-reactivity with cow's milk proteins [8–10].

2.2 Camel milk

One-humped camels (*Camelus dromedaries*) are used for many purposes such as milk, meat and wool production but they are also commonly used for transportation and agriculture works [11]. Camels are in general considered as being more environmental friendly animals when comparing with ruminants [11]. This is mainly due to the fact that camels require lower food intake, they have ability to adapt to any climate, and produce less methane than ruminants [3,12,13].

Milk from one-humped camel is commonly used in arid areas such as Middle East, parts of Asia and Australia [14,15]. Camel milk corresponds to 0.4% of the global milk production [16]. It is used as a liquid pasteurised milk but also for the production of fermented products, cheese and ice cream [15,17,18]. Recently camel milk has gained an interest as a source of proteins for infant formula production [19,20]. This is mainly due to the differences in protein composition and low sequence identity when comparing with cow's milk proteins [21,22]. Camel milk contains comparable amount of proteins when comparing with cow's milk i.e. 3.1% and 3.4% respectively [22], with a slightly different ratios between caseins and whey proteins, 24:76 and 20:80 respectively [16]. Camel milk lacks β-lactoglobulin (BLG), the major whey protein in cow's milk, while α-lactalbumin is the major whey protein in camel milk [23]. Camel milk has been debated as a candidate for CMA management because of some evidences showing a low cross-reactivity between cow's and camel milk proteins based on the evaluation of blood from patients with CMA

and due to the great tolerability of camel milk in majority of patients with CMA [24–26]. Cross-reactivity between cow's and camel milk proteins was overall studied to be low, yet, some individuals with CMA still reacted to camel milk [25,26]. Processing such as enzyme hydrolysis is commonly used for cow's milk proteins allergenicity alteration [27], and heat treatment is under research interest for its usability to alter proteins allergenicity [28]. Enzyme hydrolysis and heat treatment are therefore processing methods that could possibly alter allergenicity of camel milk proteins. However, at present there are no studies evaluating how enzyme hydrolysis and heat treatment influence camel milk proteins, from the perspective of their immunogenicity, sensitising capacity and cross-reactivity with cow's milk proteins.

Currently camel milk is used as a source of proteins in a commercially available, stage three toddler formula in the Middle East [29]. The knowledge whether camel milk could be used for the production of hypoallergenic infant formula for CMA management and whether processes such as enzyme hydrolysis and heat treatment could be applied for further reduction of camel milk allergenicity would be of a high benefit.

2.3 Animal models

Animal models are in vivo methods commonly used for initial evaluation of proteins sensitising capacity, immunogenicity, cross-reactivity and their potential tolerogenic properties. Animal models are primarily based on rodents: mice and rats and particularly breeds such as C3H/HeJ, C57BL/6 and BALB/c mice as well as Brown Norway (BN) rats as they resemble atopic individuals, being high IgE responders [30,31]. In rodents similarly as in humans, tolerance to proteins is a default immunological response after oral exposure to proteins. Therefore, in animal models, routes of administration such as intraperitoneal (i.p.), intragastric (i.g.) or epicutaneus (e.c.) are required for allergic response induction [32–34]. Yet, some require use of adjuvants for example cholera toxin (CT) from *Vibrio cholera* for i.g. route of administration to develop Th2 immune response [35]. Using animal model enables to evaluate in vivo and ex vivo end points, taking into account both cellular and humoral responses [36].

Using an animal model for initial evaluation of proteins immunogenicity and allergenicity and their potential cross-reactivity with other proteins could give a basis knowledge on how to approach further clinical studies. That could be also beneficial for camel milk as a potential alternative for CMA management. As rodents' milk does not contain BLG [37], one of the most allergic protein in cow's milk, rodents are highly valuable animal models for studying CMA.

2.4 Objectives

There are several possibilities available for CMA management in non-exclusively breastfeed infants such as different hypoallergenic infant formulas [38], while at present there are no recommendations for the use of any particular infant formula for CMA prevention [39]. Hypoallergenic infant formulas are mainly based on extensively hydrolysed cow's milk proteins and for severe CMA symptoms, amino acid-based infant formulas as well as hydrolysed rice

proteins-based formulas are considered as the second choice of CMA management [6]. Sources of proteins for potential, future infant formulas manufacture based on other than cow's milk mammalian milk proteins are currently under investigation.

Camel milk has recently gained an interest as a potential source of proteins in infant formula manufacture from the perspective of CMA management and prevention. Using a well-estabilished BN rat model, the aims of this project were to (**1**) investigate immunogenicity, allergenicity and cross-reactivity of intact cow's and camel milk proteins, (**2**) investigate how processing such as heat treatment and enzyme hydrolysis influenced cow's and camel milk protein physicochemical properties, (**3**) investigate immunogenicity, sensitising and cross-reactive capacity of cow's and camel milk processed proteins, (**4**) investigate whether intact camel milk can prevent CMA and whether cow's milk can prevent camel milk allergy and finally using human samples, (**5**) to investigate how patients with confirmed CMA reacted to cow's and camel milk processed products.

This was investigated by inducing sensitisation towards intact cow's and camel milk as well as cow's milk whey and casein fraction (**Manuscript II**) in BN rat model. In addition, enzyme hydrolysed (EH) and heat treated (HT) cow's and camel milk were first prepared and analysed for their physiochemical properties (**Manuscript III**) and further sensitisation towards intact, EH and HT cow's and camel milk was induced (**Manuscript IV**) in BN rat model. The capacity of cow's and camel milk to prevent cow's and camel milk allergy were investigated in a well-estabilished prophylactic BN rat model (**Manuscript V**). Finally, serum or plasma samples from children with confirmed CMA were investigated for their antibodies reactivity towards intact, EH and HT cow's and camel milk (**Manuscript VI**).

3 Manuscript II

Comparison of the Allergenicity and Immunogenicity of Camel and Cow's Milk-A Study in Brown Norway Rats

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Comparison of the Allergenicity and Immunogenicity of Camel and Cow's Milk—A Study in Brown Norway Rats

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Abstract: Background: When breastfeeding is impossible or insufficient, the use of cow's milk-based hypoallergenic infant formulas is an option for infants suffering from or at risk of developing cow's milk allergy. As the Camelidae family has a large evolutionary distance to the Bovidae family and as camel milk differs from cow's milk protein composition, there is a growing interest in investigating the suitability of camel milk as an alternative to cow's milk-based hypoallergenic infant formulas. Methods: The aim of the study was to compare the allergenicity and immunogenicity of camel and cow's milk as well as investigating their cross-reactivity using a Brown Norway rat model. Rats were immunised intraperitoneally with one of four products: camel milk, cow's milk, cow's milk casein or cow's milk whey fraction. Immunogenicity, sensitising capacity, antibody avidity and cross-reactivity were evaluated by means of different ELISAs. The eliciting capacity was evaluated by an ear swelling test. Results: Camel and cow's milk showed similarity in their inherent immunogenicity, sensitising and eliciting capacity. Results show that there was a lower cross-reactivity between caseins than between whey proteins from camel and cow's milk. Conclusions: The study showed that camel and cow's milk have a low cross-reactivity, indicating a low protein similarity. Results demonstrate that camel milk could be a promising alternative to cow's milk-based hypoallergenic infant formulas.

Keywords: food allergy; cow's milk; camel milk; infant formula; animal models

1. Introduction

Cow's milk allergy (CMA) is the most prevalent food allergy in infants and small children [1], affecting around 2.5% [2,3], although differences are observed between studies and countries [4]. Although most CMA children outgrow their allergy, some keep it for life [5]. Originally, it was though that most children did outgrow their CMA before the age of three years, but there seems to be a tendency that more and more children outgrow their CMA later in life and for some it may even last for lifetime [6,7]. Breastfeeding is the most suited source of nutrition for a newborn infant [8]. However, in some situations, breastfeeding is impossible or insufficient and a substitute such as an infant formula is needed [9]. Infant formulas are usually based on cow's milk, as this is the most easily accessible milk source globally [10]. When an infant suffers from or is at risk of developing CMA, alternatives to conventional infant formulas are recommended such as hypoallergenic infant formulas, based on extensively or partially hydrolysed cow's milk proteins [11]. In addition to cow's milk-based hypoallergenic infant formulas, additional alternatives to conventional infant formulas are found on the market, such as amino acid-based infant formulas, plant-based infant formulas

(e.g., soya-based) and infant formulas based on other mammalian milk (e.g., goat or sheep) [8,12,13]. Extensively and partially hydrolysed infant formulas as well as amino acid-based infant formulas are poor in flavour, and, thus, some newborns may refuse them [5,14]. On the other hand, it has been reported that sheep and goat milk-based infant formulas may only be an alternative for some newborns due to a high cross-reactivity between cow's milk proteins and proteins from goat and sheep milk [13,15]. In addition, plant-based infant formulas are seldom recommended due to their low nutritional value [16,17]. For those reasons, new or improved alternatives to conventional infant formulas are still of interest.

Due to the large evolutionary distance between Camelus dromedaries (Camelidae family) and the Bovidae family animals, camel milk is quite different in its composition compared to cow's milk. Equivalent to human milk, the allergenic milk protein β-lactoglobulin (BLG) is also absent in camel milk [18]. Moreover, similar to human milk, camel milk has approximately double the amount of β-casein and approximately five times the amount of immunoglobulins in comparison to cow's milk [19]. Rastani et al. [13] showed that CMA patients did not recognise camel milk by immunoblotting and concluded that camel milk is a promising alternative to cow's milk for infant formula manufacture. Further, based on double-blind, placebo-controlled food challenges, Navarre-Rodriguez et al. [20] concluded that camel milk is a safe and tolerable alternative for CMA patients above the age of one year. Camel milk is already commercially available in the Middle East, Australia, United Kingdom and the Netherlands [21–24]. In other regions such as in African countries, it is a traditionally consumed product, although without a control on its quality and safety [25]. There are a number of studies showing that camel milk is nutritionally suitable for human consumption [21,26]. For those reasons, camel milk is an exciting and suitable product with the potential to be a future alternative to hypoallergenic cow's milk-based infant formulas in prevention, treatment and management of CMA in infants and small children.

The purpose of this study was to investigate the immunogenicity and allergenicity of camel and cow's milk as well as studying cross-reactivity between proteins from the two sources. To do this, Brown Norway (BN) rats were immunised intraperitoneally (i.p.) with either camel milk, cow's milk, cow's milk casein fraction or cow's milk whey fraction and antibody responses were evaluated for level, specificity, avidity, functionality and cross-reactivity by means of different enzyme-linked immunosorbent assays (ELISAs), immunoblotting and in vivo test. This should allow for an overview of the usability of camel milk as an alternative to hypoallergenic infant formulas.

2. Materials and Methods

2.1. Products

Powders of cow's milk, cow's milk casein fraction and cow's milk whey fraction were kindly provided by Arla Foods Ingredients Videbæk, Denmark. Powder of camel milk was kindly provided by Dairy Farm Smits, Berlicum, the Netherlands. Products were tested by Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (88282, Thermo Fisher, Waltham, MA, USA) in accordance with the instruction given by the manufacturer. Whereas camel milk, cow's milk and cow's milk whey fraction had an endotoxin level <2 endotoxin units (EU) per mg of protein, cow's milk casein fraction had an endotoxin level of approximately 66 EU per mg of protein.

2.2. In Silico Protein Analyses

CLC Main Workbench 8.0 (Redwood City, CA, USA) was used to compare selected protein amino acid sequences from cow's milk with those of goat, sheep, camel and human milk. Protein sequences were downloaded from UniProt (http://www.uniprot.org).

2.3. Denaturation of Products

Camel milk and cow's milk were denatured to obtain unfolded structures of proteins. Denaturation was performed by reduction and alkylation, as previously described by Madsen et al. [27].

2.4. SDS-PAGE Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with camel milk, denatured camel milk, cow's milk, denatured cow's milk, cow's milk casein fraction and cow's milk whey fraction was performed using 5 µg of each product dissolved in Laemmli buffer (65.8 mM Tris-HCl, pH 6.8, 26.3% (*w*/*v*) glycerol and 2.1% (*w*/*v*) SDS, 161-0737, Bio-Rad, Hercules, CA, USA) with addition of β-mercaptoethanol (14.2 M, 161-0710, Bio-Rad). Samples were incubated for 5 min at 95 °C and afterwards loaded onto a 4–20% gel (Mini-Protean TGX Stain-Free gel, 456-8093, Bio-Rad). SDS-PAGE was performed in running buffer (25 mM Tris and 192 mM Glycine and with addition of 0.1% (*w*/*v*) SDS, pH 8.3, 161-0732, Bio-Rad). Additionally, 10 µL of the molecular weight Precision Plus Protein™ Unstained Standard (161-0363, Bio-Rad) was loaded onto the gel. Gel electrophoresis was run at 200 V with constant current at room temperature (RT). Afterwards, the gel was stained with Bio Safe™ Coomassie (161-0786, Bio-Rad) for 1 h at RT and photographed using Imager ChemiDoc XRS+ (Bio-Rad).

2.5. Animals

BN rats were from the in-house breeding colony, at the National Food Institute, Technical University of Denmark, Denmark, and kept in macrolon cages at 22 °C ± 1 °C with 55 ± 5% relative humidity at a 12-h light–dark cycle. Air exchange was applied 8–10 times per hour with overpressure. BN rats were inspected twice a day and weighted once per week. Rats were kept on a diet free from milk and soy allergens for ≥10 generations. Feed containing rice flour and fish was given ad libitum as well as was acidified tap water.

2.6. Animal Sensitisation Studies

To sensitise animals and raise antibodies against camel milk, cow's milk, cow's milk casein fraction or cow's milk whey fraction, BN rats 4–7 weeks of age, were divided into five groups of eight rats (n = four/gender), and housed two per cage. Groups of rats were immunised i.p. three times with 200 µg of product dissolved in phosphate buffer saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) without the use of adjuvant one time at Day 0, 14 and 28 (Figure 1). One group of rats was not immunised to act as a control group (naïve animals) for an ear swelling test. At Day 35, rats were sacrificed and blood collected. The animal experiment was carried out at the National Food Institute, Technical University of Denmark under ethical approval given by the Danish Animal Experiments Inspectorate and the authorisation number 2015-15-0201-00553-C1. The experiment was overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

2.7. Ear Swelling Test

To investigate the eliciting capacity of camel and cow's milk, at Day 33 of the experiment, an ear swelling test was performed. Rats were anesthetised with hypnorm-dormicum and baseline ear thickness was measured. Subsequently, 20 µL of PBS with 10 µg of camel milk or 10 µg of cow's milk were injected into the right or left ear, respectively, and ear thicknesses were measured again one hour after injections. Naïve rats were included to see unspecific ear swelling and irritation capacity after camel and cow's milk protein ear injection. Delta ear swelling was calculated.

Figure 1. Animal experimental design. Brown Norway rats were immunised i.p. with 200 µg of camel milk, cow's milk, cow's milk casein fraction or cow's milk whey fraction three times, at Days 0, 14 and 28. At Day 33 an ear swelling test was performed and at Day 35 rats were sacrificed and blood collected. Pictures were purchased from https://www.colourbox.com.

2.8. Indirect ELISA for Specific IgG1 Detection

To detect IgG1 antibodies specific for camel milk, denatured camel milk, cow's milk and denatured cow's milk, indirect ELISAs were performed using Maxisorp microtitre plates (96-well, Nunc, Roskilde, Denmark). Plates were coated with 100 µL/well of 10 µg/mL of camel milk, denatured camel milk, cow's milk or denatured cow's milk, in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), and incubated overnight at 4 °C. Between each step, plates were washed five times in PBS with 0.01% (*w*/*v*) Tween 20 (PBS-T). For all steps that required incubation, plates were incubated for one hour in the dark at RT, with gentle agitation. First, plates were incubated with 50 µL/well of two-fold serial dilution of serum samples (*v*/*v*) in PBS-T. In each plate, positive and negative control serum samples were included in order to identify potential plate-to-plate variance. For antibody detection, 50 µL/well of secondary antibody (horse radish peroxidase (HRP)-labelled-mouse-anti-rat IgG1, 3060-05, Southern Biotech, Birmingham, AL, USA) diluted 1:20,000 (*v*/*v*) in PBS-T was added to the plates. After incubation plates were additionally washed twice with tap water. To visualise specific antibody detection, 100 µL/well of TMB-one (3,3′,5,5′-tetramethylobenzidine, 4380A, Kementec Diagnosis, Taastrup, Denmark) was added and incubated for 12 min at RT. The reaction was stopped with 100 µL/well 0.2 M H_2SO_4 and the absorbance was measured at 450 nm with a reference wavelength of 630 nm using a microtitre reader (Gen5, BioTek, EL800 Instrument, Winooski, VT, USA). The cut-off values were set to be higher than the mean absorbance of negative control plus three times the standard deviation (SD). Results were expressed in log2 titre values with a cut-off at the optical density (OD) of 0.1 for IgG1 specific for camel milk, cow's milk and denatured cow's milk and 0.15 for IgG1 specific for denatured camel milk.

2.9. Antibody Capture ELISA to Detect Specific IgE

To detect IgE specific for camel milk, denatured camel milk, cow's milk and denatured cow's milk, antibody capture ELISAs were performed using Maxisorp microtitre plates (96-well, Nunc) coated with 100 µL/well of mouse anti-rat IgE (HDMAB-123, Hydri-Domus, Nottingham, UK) diluted 1:2000 in coating buffer and incubated overnight at 4 °C. Between each step, plates were washed five times with PBS-T. For all steps that required incubation, plates were incubated for one hour in the dark at RT, with gentle agitation. For camel and cow's milk specific IgE detection, antibody capture ELISA was optimised to use proper blocking for each product. Plates were blocked at 37 °C, 200 µL/well, with 3% (*v*/*v*) horse serum for camel milk specific IgE detection and 5% (*v*/*v*) rabbit serum for cow's milk specific IgE detection, diluted in PBS-T. Subsequently, plates were incubated for one hour with 50 µL/well of two-fold serial dilution of serum samples (*v*/*v*) in PBS-T. In each plate, positive and negative control serum samples were included. Afterwards, 50 µL/well of 0.05 µg/mL of 10:1 digoxigenin (DIG)-coupled camel milk or 0.1 µg/mL of 10:1 DIG-coupled cow's milk in PBS-T

were added, to detect specific IgE. Next, plates were incubated with 100 µL/well of HRP-labelled sheep-anti-DIG-POD (11633716001, Roche, Diagnostics GmbH, Mannheim, Germany) diluted 1:1000 (*v*/*v*) in PBS-T. After this step, plates were additionally washed twice with tap water and incubated for 12 min with 100 µL/well of TMB-one (Kementec Diagnosis). The reaction was stopped with 100 µL/well of 0.2 M H_2SO_4 and the absorbance was measured. Results were expressed as log2 titre value with an individual cut-off of plates at an OD of 0.145–0.2 for IgE specific for camel milk and of 0.125–0.175 for IgE specific for cow's milk.

2.10. Avidity Measurements

To measure binding strength between antigens and IgG1 antibodies from serum samples, avidity ELISA was performed as previously described by Bøgh et al. [28]. Serum samples from rats that reached an OD of at least 0.5 were included.

2.11. Inhibitory ELISA

To examine the cross-reactivity between proteins from camel and cow's milk, inhibitory ELISA was performed. The procedure was as described for the indirect IgG1 ELISA with few exceptions. Serum samples for each group of animals were pooled and diluted in PBS-T to reach an OD of approximately 2.0. Serum pools were then pre-incubated for one hour with ten-fold serial dilutions of camel and cow's milk. After pre-incubation, samples were added to the plates in duplicates and incubated for one hour. The assay was performed twice. The results were expressed in percentage inhibition against the concentration of the inhibitor.

2.12. Immunoblotting

To do immunoblotting, SDS-PAGE was performed with 5 µg of camel and cow's milk as described previously. In addition, SDS-PAGE with an eight-time higher load of proteins (40 µg) was performed to visualise cross-reactivity. After SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes (Trans-Blot® Turbo™ Mini PVDF Transfer Pack, 1704156, Bio-Rad) by semidry blotting (Trans-Blot® Turbo™ Transfer System, 170-4150, Bio-Rad) at constant 200 V. Membranes were washed three times for 5 min in PBS-T (0.05% *v*/*v* Tween 20) and each blocked with 20 mL of 5% ovalbumin (OVA, egg whites from chicken, Sigma Aldrich, St. Louis, MO, USA) diluted in PBS-T (0.1% *v*/*v* Tween 20) and incubated for one hour in the dark at RT, on a shaking table. The 5% OVA solution was used during the whole experiment as a blocking solution. After blocking, membrane was divided into two pieces, both pieces with 5 µg of camel and cow's milk. Next, 10 mL of serum pooled from rats immunised with cow's milk diluted 1:3000 (*v*/*v*) in blocking solution or serum pooled from rats immunised with camel milk diluted 1:8000 (*v*/*v*) in blocking solution were added separately to each half of the membrane containing 5 µg of camel and cow's milk and incubated for one hour in the dark at RT, on a shaking table. Half of the membrane with 40 µg of cow's milk was incubated with serum pooled from rats immunised with camel milk diluted 1:500 (*v*/*v*) in blocking solution, while the other half of the membrane with 40 µg of camel milk was incubated with serum pooled from rats immunised with cow's milk diluted 1:500 (*v*/*v*) in blocking solution. Afterwards, membranes were washed three times for 5 min in PBS-T (0.05% *v*/*v* Tween 20) and 10 mL of the secondary antibody diluted 1:15,000 together with StrepTacin-HRP conjugate (Bio-Rad) for Precision Plus Protein™ Unstained Standard detection, diluted 1:15,000 in blocking solution were added to each half of the membrane. Membranes were incubated for one hour in the dark at RT, on a shaking table. Subsequently, membranes were washed three times for 5 min in PBS-T (0.05% *v*/*v* Tween 20) followed by PBS washing two times for 5 min to remove the detergent. Membranes were incubated with peroxidase substrate (Clarity™ Western ECL Substrate, 1705060, Bio-Rad) for 5 min. After incubation, membranes were developed and photographed using Imager ChemiDoc XRS+ (Bio-Rad).

2.13. Statistical Analysis of Data

Graphs and statistical analyses of the data were performed using GraphPrism version 7.0 (San Diego, CA, USA). Results from indirect and antibody-capture ELISAs were expressed as log2 antibody titre values.

ELISA results expressed as log2 antibody titres were tested for normality distribution. Based on the results, either parametric or non-parametric *t*-tests were performed. Differences were regarded as statistically significant when $p \leq 0.05$. Asterisks indicate statistically significant differences between two given groups: $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$, $**** p \leq 0.0001$.

Inhibition curves resulting from avidity and inhibitory ELISA were examined with one-way repeated-measurements ANOVA test. Analyses showed no statistically significant differences between curves, thus IC_{50} calculations were performed. IC_{50} was calculated using sigmoidal dose response with non-linear regression.

3. Results

3.1. Protein Characterisation

The primary sequence from selected cow's milk proteins were aligned to their counterpart proteins in milk from goat, sheep, camel and human to investigate the amino acid sequence identity between the different species and to predict the potential cross-reactivity between proteins of interest. As shown in Table 1, goat and sheep milk protein sequences show a very high percentage identity to cow's milk proteins, ranging from a protein sequence identity of 85–95% for goat and sheep. A much lower protein sequence identity was evidenced between camel and cow's milk proteins, where the protein identity ranged from 47% to 81%. This is very similar to the protein sequence identity of human and cow's milk proteins ranging from 33% to 76% and human and camel milk proteins ranking from 40% to 76%. In addition, neither camel nor human milk contains BLG [18]. Similarities between camel and cow's milk caseins sequences were shown to be slightly lower than between the whey proteins.

Table 1. Amino acid sequence identity between cow's and goat, sheep, camel and human milk proteins.

		Goat	Sheep	Camel	Human [(c)]
Casein	β-casein	91	91	67	55 (60)
	αs1-casein	88	88	47	33 (40)
	αs2-casein	88	89	56	NA [(a)]
	κ-casein	85	85	58	52 (60)
Whey	α-lactalbumin	95	95	60	74 (62)
	β-lactoglobulin	93	93	NA [(b)]	NA [(b)]
	serum albumin	88	92	81	76 (76)
	lactoferrin	92	92	75	70 (74)

Sequence identity (%) between selected cow's milk proteins and their counterpart milk proteins from goat, sheep, camel and human expressed in percentage. Sequence alignments were performed using CLC Main Workbench 8.0 and UniProt and NCBI database. NA: not available. (a) αs2-casein not identified in human milk [29,30]. (b) β-lactoglobulin not available in camel and cow's milk [18]. (c) Numbers in brackets represents sequence identity between human and camel milk. Accession number: β-casein: Cow: AAA30431; Goat: AAA30906; Sheep: CAA56139; Camel: CDO50354; Human: AAC82978. αs1-casein: Cow: AAA30429; Goat: CAA51022; Sheep: AEN84772; Camel: O97943; Human: CAA55185. αs2-casein: Cow: NP_776953; Goat: CAC21704; Sheep: CAA26983; Camel: O97944. κ-casein: Cow: CAA33034; Goat: CAA43174; Sheep: NP_001009378; Camel: CCI79378; Human: CAA47048. α-lactalbumin: Cow: CAA29664; Goat: CAA28797; Sheep: CAA29665; Camel: P00710; Human: AAA60345. β-lactoglobulin: Cow: CAA32835; Goat: CAA79623; Sheep: CAA31305. serum albumin: Cow: CAA41735; Goat: XP_005681801; Sheep: CAA34903; Camel: XP_010981066; Human: AAN17825; lactoferrin: Cow: AAA30610; Goat: AAA97958; Sheep: ACT76166; Camel: CAB53387; Human AAA59511.

SDS-PAGE electrophoresis was performed to display the protein profile of the products used in this study. Caseins run as thick bands between 25 and 37 kilodalton (kDa) in both camel and cow's milk (Lanes 1–4, Figure 2) as well as in the casein fraction of cow's milk (Lane 5) [31]. The band corresponding to a molecular weight (MW) of around 30 kDa represents β-casein while the band

immediately above represents α-caseins with a MW of around 35 kDa [19,31]. In cow's milk as well as in the whey fraction of cow's milk (Lanes 3 and 6), a clear band representing BLG is evident (~18 kDa) [1], which is not present in camel milk. In all lanes except for the lane corresponding to the casein fraction of cow's milk (Lane 6), the lower band represents α-lactalbumin (ALA) (~14 kDa), while the two upper bands most likely represent lactoferrin (LF) (~75 kDa) and serum albumin (SA) (~66 kDa) [19]. Immunoglobulins (~150 kDa) are only hardly seen due to their low amount in the milk products. LF and SA are slightly more visible in the denatured version of the milk products (Lanes 2 and 4) than their native counterparts. Another difference between the native and denatured version of the milk products are a lower mobility of proteins in the denatured versions compared to the native versions.

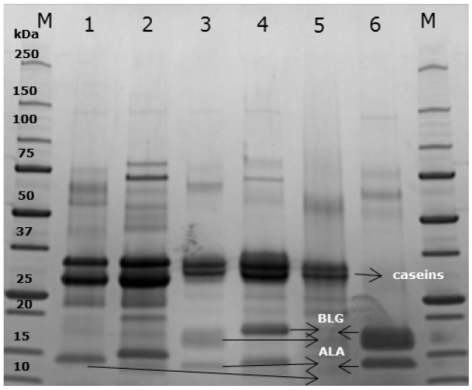

Figure 2. SDS-PAGE electrophoresis. Gel electrophoresis, with native and denatured camel and cow's milk as well as with native cow's milk casein fraction and native cow's milk whey fraction, was performed to display protein profiles. M, protein standard (kDa); 1, camel milk; 2, denatured camel milk; 3, cow's milk; 4, denatured cow's milk; 5, cow's milk casein fraction; 6, cow's milk whey fraction. BLG, β-lactoglobulin; ALA, α-lactalbumin.

3.2. Camel and Cow's Milk Immunogenicity and Cross-Reactivity

Serum samples from individual BN rats immunised with camel milk, cow's milk, cow's milk casein fraction or cow's milk whey fraction were assessed for specific IgG1 by means of indirect ELISAs. Figure 3A shows the IgG1 responses against both the native and denatured version of camel as well as cow's milk proteins.

The immunogenicity of camel and cow's milk appears to be very similar as there is no statistically significant difference between the level of camel milk specific IgG1 raised against camel milk and the level of cow's milk specific IgG1 raised against cow's milk (Figure 3A). For both antibodies raised against camel or cow's milk proteins, there is a statistically significant difference between the IgG1 reactivity towards camel milk and cow's milk proteins, indicating a low cross-reactivity between camel and cow's milk proteins. For antibodies raised against camel milk, the IgG1 reactivity against cow's milk proteins was ~30 fold lower than the reactivity against camel milk proteins, measured by the amount of specific antibodies. Opposite the IgG1 reactivity against camel milk proteins was ~50-fold lower than the reactivity against cow's milk proteins for sera raised against cow's milk proteins. This was shown irrespectively of responses that were measured against the native or denatured version of the milk proteins.

The IgG1 responses in rats immunised with either the casein or the whey fraction of cow's milk, are shown in Figure 3B. For both groups of animals, the IgG1 responses against native and denatured camel milk were statistically significantly lower than the responses against native and denatured cow's

milk, stressing a low cross-reactivity for both the casein and the whey fraction of camel and cow's milk proteins. For antibodies raised against casein, the IgG1 reactivity against native camel milk proteins was ~250-fold lower than the reactivity against cow's milk proteins, while for antibodies raised against whey, the IgG1 reactivity against camel milk proteins was ~15-fold lower than the reactivity against cow's milk proteins. This indicates a lower cross-reactivity between camel and cow's milk caseins than whey proteins.

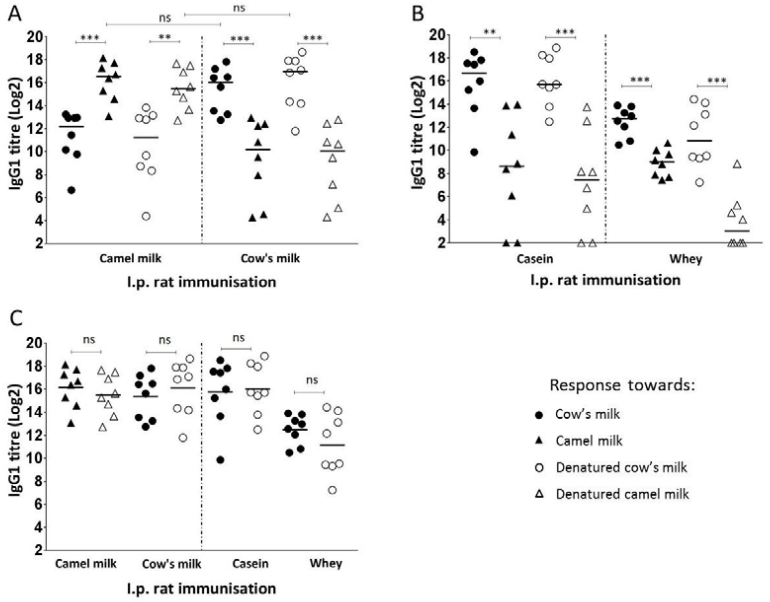

Figure 3. Specific IgG1 antibody responses. Comparison of specific IgG1 antibody responses toward cow's milk (●), camel milk (▲), denatured cow's milk (○) and denatured camel milk (Δ) raised in rats immunised with camel milk, cow's milk, cow's milk casein fraction or cow's milk whey fraction. Each symbol represents the specific IgG1 titre value for an individual rat. (**A**) Comparison of native and denatured camel milk and cow's milk specific IgG1 antibody responses in rats immunised with camel or cow's milk, respectively. Horizontal lines display the median values for each group of rats. Statistically significant difference between two groups was determined using the non-parametric Mann–Whitney test. Asterisks indicate statistically significant differences between the two given groups when: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** $p \leq 0.0001$. (B) Comparison of native and denatured camel milk and cow's milk specific IgG1 antibody responses in rats immunised with cow's milk casein or whey fraction. Horizontal lines display the median values for each group of rats. Statistically significant difference between two groups was determined using the non-parametric Mann–Whitney test. Asterisks indicate statistically significant differences between the two given groups when: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** $p \leq 0.0001$. (C) Comparison of IgG1 antibody reactivity against native vs. denatured camel and cow's milk. Horizontal lines display the mean values for each group of rats. Statistically significant difference between two groups was determined using the parametric *t*-test. Asterisks indicate statistically significant differences between the two given groups when: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** $p \leq 0.0001$.

3.3. Linear and Conformational Epitope Recognition

The study showed that there were no statistically significant differences between the IgG1 responses against the native and denatured versions of milk proteins for rats immunised with neither

camel milk nor cow's milk, indicating that linear epitopes are dominating both responses (Figure 3C). In addition, the IgG1 raised against cow's milk caseins showed no statistically significant difference in their reactivity against the native or denatured version of milk proteins with an approximate ratio of 1:1. In contrast, although IgG1 raised against cow's milk whey showed no statistically significant difference in their reactivity against the native and denatured version of milk proteins, the ratio between IgG1 specific for native vs. denatured cow's milk was 4:1. This demonstrates that, while caseins primarily induce antibodies against linear epitopes, whey primarily induces antibodies against conformational epitopes.

3.4. Inhibitory ELISA

Inhibitory ELISA was performed with sera pools from groups of rats immunised with camel milk, cow's milk, cow's milk casein or whey fraction in order to evaluate the competitive capacity of native as well as denatured camel and cow's milk.

3.4.1. IgG1 Antibody Competition of Native Camel and Cow's Milk

While native camel milk was able to fully inhibit antibodies raised against camel milk, native cow's milk was only able to inhibit ~50% of the antibodies raised against camel milk (Figure 4A). On the other hand, while native cow's milk was fully capable of inhibiting the antibodies raised against cow's milk, native camel milk was only capable of inhibiting ~35% of antibodies raised against cow's milk (Figure 4B). While native cow's milk was able to fully inhibit antibodies raised against both the casein and the whey fraction of cow's milk, native camel milk was only able to inhibit ~30% of antibodies raised against cow's milk casein (Figure 4C) and ~45% of antibodies raised against cow's milk whey (Figure 4D). This confirms previous results, showing a lower cross-reactivity between casein compared to the whey fraction of camel and cow's milk.

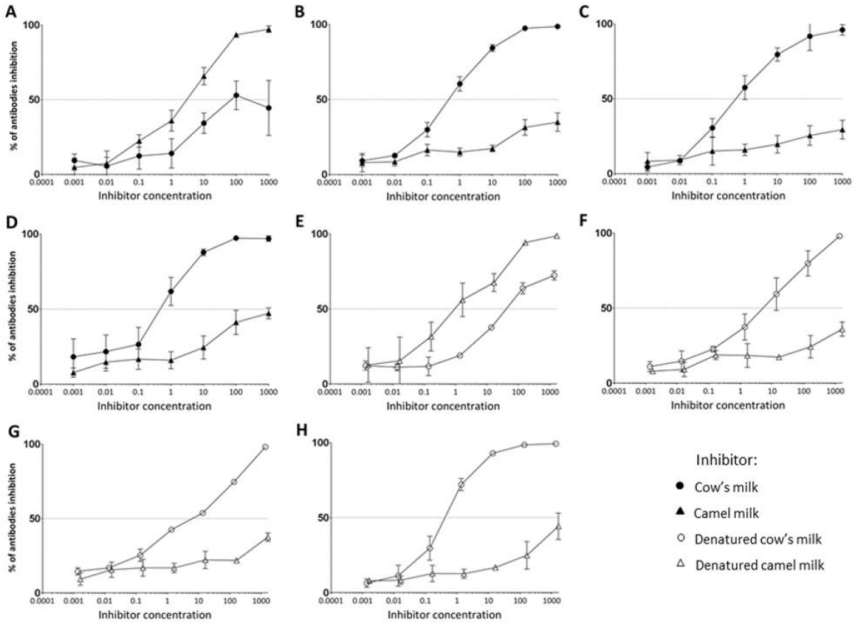

Figure 4. IgG1 antibody binding competition. Inhibitory ELISA with native (●) or denatured (○) cow's milk or native (▲) or denatured (Δ) camel milk as inhibitors was performed using serum pools from rats immunised with camel milk, cow's milk, cow's milk casein fraction or cow's milk whey fraction.

Each symbol represents the percent inhibition of IgG1 specific antibodies at different inhibitor concentrations. Error bars in the inhibition curves represent ± standard deviation (SD). (**A**) Inhibition curve for sera raised against camel milk. (**B**) Inhibition curve for sera raised against cow's milk. (**C**) Inhibition curve for sera raised against cow's milk casein fraction. (**D**) Inhibition curve for sera raised against cow's milk whey fraction. (**E**) Inhibition curve for sera raised against linear epitopes of camel milk. (**F**) Inhibition curve for sera raised against linear epitopes of cow's milk. (**G**) Inhibition curve for sera raised against linear epitopes of cow's milk casein fraction. (**H**) Inhibition curve for sera raised against linear epitopes of cow's milk whey fraction.

3.4.2. IgG1 Antibody Competition Towards Denatured Camel and Cow's Milk

By performing inhibitory ELISA with the use of denatured versions of camel and cow's milk, we could only study the cross-reactivity as a measure of antibodies raised against linear epitopes. While denatured camel milk was able to inhibit fully antibodies raised against linear epitopes of camel milk, denatured cow's milk was able to inhibit ~70% of the antibodies raised against linear epitopes of camel milk (Figure 4E). On the other hand, while denatured cow's milk was fully capable of inhibiting the antibodies raised against linear epitopes of cow's milk, denatured camel milk was only capable of inhibiting ~35% of antibodies raised against cow's milk (Figure 4F). While denatured cow's milk was able to fully inhibit antibodies raised against both linear epitopes of the casein and the whey fraction of cow's milk, denatured camel milk was only able to inhibit ~35% of antibodies raised against linear epitopes of cow's milk casein (Figure 4G) and ~45% of antibodies raised against linear epitopes of cow's milk whey (Figure 4H). This indicated a slightly higher cross-reactivity between linear epitopes compared to conformational epitopes of camel and cow's milk.

3.5. Specific IgG1 Antibody Avidity

Avidity ELISAs were performed to evaluate binding strength between specific IgG1 antibodies and the milk proteins. Figure 5 displays the amount of potassium thiocyanate (KSCN) needed to inhibit 50% of the antibody–antigen binding. Results indicated that there were no statistically significant differences in binding strength between IgG1 raised against camel milk and camel milk or cow's milk. Similar results were shown for IgG1 raised against cow's milk and their binding strength towards cow's milk and camel milk, although slightly higher avidity was shown between antibodies raised against cow's milk and cow's milk compared to the avidity between antibodies raised against cow's milk and camel milk.

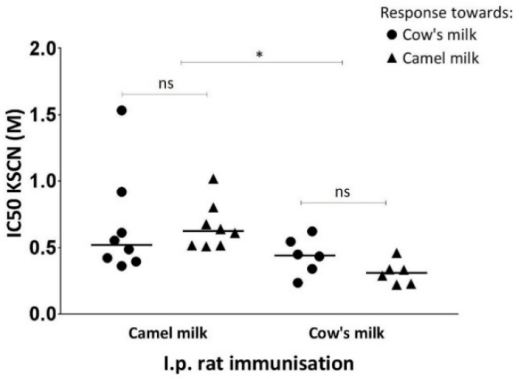

Figure 5. Avidity of IgG1 specific for cow's milk (●) or camel milk (▲). Serum samples from rats immunised with camel milk or cow's milk were evaluated to compare specific IgG1 antibody binding strength towards camel or cow's milk. Each symbol represents an individual rat. The avidity is expressed

as potassium thiocyanate concentration needed to inhibit 50% of the IgG1 response towards camel or cow's milk for groups of rats immunised with camel milk or cow's milk. Horizontal lines display the median values for each group of rats. Statistically significant difference between two groups was determined using the non-parametric Mann–Whitney test. Asterisks indicate statistically significant differences between two given groups when: $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$, $**** p \leq 0.0001$.

3.6. Sensitising Capacity of Camel and Cow's Milk

As IgE is the main player in food allergies [32], specific IgE titres were determined by the use of antibody-capture ELISAs. The results showed no obvious differences in the sensitising capacity of camel and cow's milk proteins, both products containing the capacity to induce high levels of specific IgE antibodies (Figure 6). No statistical analysis could be performed as the camel and cow's milk assays cannot be directly compared because of their potential different sensitivity. In line with the specific IgG1 responses, also for the specific IgE responses a low cross-reactivity between camel and cow's milk proteins was identified. Furthermore, in accordance with the IgG1 results, also for the IgE results a lower cross-reactivity could be observed for the casein fraction compared to the whey fraction of camel and cow's milk proteins.

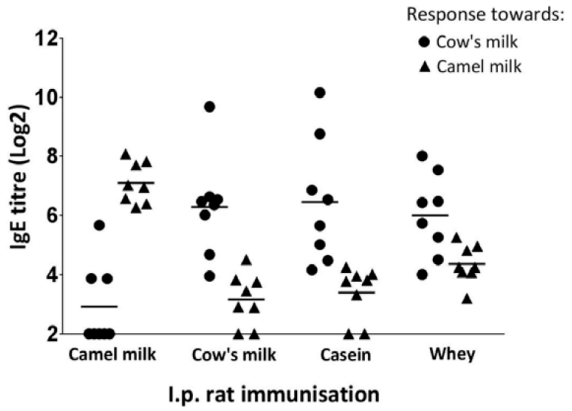

Figure 6. Specific IgE antibody responses. Comparison of specific IgE responses towards cow's (●) and camel milk (▲) in rats immunised with camel milk, cow's milk, cow's milk casein fraction or cow's milk whey fraction. Each symbol represents a specific IgE titre value for an individual rat. Horizontal lines on the graph display the median values for each group of rats.

3.7. Eliciting Capacity of Camel and Cow's Milk

The ability of camel and cow's milk to elicit allergic reactions was determined by an ear swelling test (Figure 7). Rats sensitised to camel milk showed a larger reaction towards camel milk than towards cow's milk, and opposite rats sensitised to cow's milk showed a larger reaction against cow's milk than camel milk, which correlates very well with the specific IgE responses (Figure 6), and confirms the low cross-reactivity between camel and cow's milk proteins. While a statistically significant difference was obtained for rats sensitised with camel milk, no statistically significant difference was obtained for rats sensitised with cow's milk. This may be explained by the fact that only seven animals are included in the cow's milk sensitised group compared to eight animals in the camel milk sensitised group, as one cow's milk sensitised animal died during the ear swelling test due to anaphylaxis. This has biased the results as this animal would probably be the one that would have responded with the greatest ear swelling. The groups immunised with cow's milk casein fraction or cow's milk whey fraction both

showed a significantly larger response towards cow's milk compared to camel milk; however, in line with the antibody responses, the casein proteins were shown to have a lower cross-reactivity than the whey proteins.

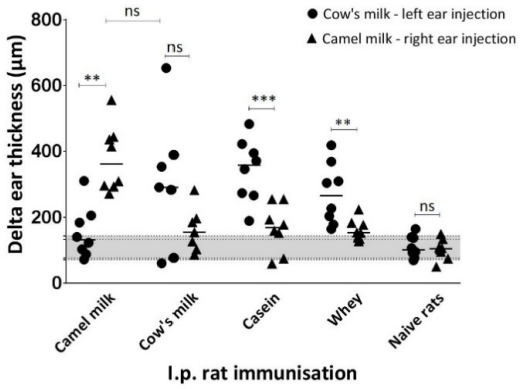

Figure 7. IgE functionality. Comparison of eliciting capacity of camel and cow's milk measured by an ear swelling test in rats immunised with camel milk, cow's milk, cow's milk casein fraction or cow's milk whey fraction. Delta ear thicknesses was calculated based on differences in ear thickness before and one hour after the ear injection of cow's milk solution to the left ear (●) and camel milk solution to the right ear (▲) at Day 33. Each symbol represents the delta ear thickness for an individual rat. Horizontal lines on the graph display the median values for each group of rats. Naïve rats correspond to the control group and define the median delta ear thickness with SD (grey coloured area) indicating no elicitation but an ear swelling caused by the injection volume. Statistically significant difference between two groups was determined using the non-parametric Mann–Whitney test. Asterisks indicate statistically significant differences between two given groups when: $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$, $**** p \leq 0.0001$.

3.8. Immunoblot

Immunoblotting was performed to investigate the specificity of the responses towards camel and cow's milk. Figure 8A shows the specificity of antibodies raised against cow's milk. BLG (~18 kDa) and the two casein fractions between 25 and 37 kDa were the proteins that antibodies specific for cow's milk reacted most pronounced to. Moreover, a hardly visible band was seen between 50 and 75 kDa indicating a weak reactivity towards SA (~66 kDa). There was no visible reaction of antibodies specific for cow's milk for camel milk proteins. The opposite situation is shown in Figure 8B where the specificity of antibodies raised against camel milk was evaluated. Here, antibodies reacted most pronounced with the camel milk β-casein fraction seen between 25 and 37 kDa standard marker bands, while there was no detectable reaction towards cow's milk proteins. However, the pooled serum dilutions used for the immunoblots were high, with a dilution of 1:3000 for sera raised against cow's milk and 1:8000 for sera raised against camel milk, for which reasons only the proteins with the strongest IgG1 binding capacity were visualised. As the ELISA assay showed very low cross-reactivity between camel and cow's milk proteins, we decided to use eight times higher protein concentration and lower serum pools dilution in order to visualise the proteins responsible for the cross-reactivity. The dilution used for both camel and cow's milk raised sera was 1:500. Figure 8C,D show cross-reactivity between camel and cow's milk proteins. Antibodies specific for cow's milk were able to cross-react exclusively with camel milk whey proteins. There were visible bands between 50 and 75 kDa indicating the most pronounced cross-reactivity with camel milk SA (~66 kDa) and LF (~75 kDa). Another weak but visible band was detected around 15 kDa indicating a very low cross-reactivity with camel milk ALA (Figure 8C). Another very weakly detectable band was at

approximately 150 kDa. This probably corresponded to immunoglobulins [33]. Antibodies specific for camel milk showed a very weak reaction with cow's milk caseins between the 25 and 37 kDa standard marker bands, and with a whey protein appeared between the 50 and 75 kDa standard marker bands (Figure 8D).

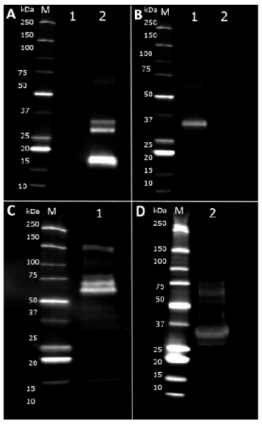

Figure 8. Immonublotting with camel and cow's milk. M, 2 µL of Protein Standard (kDa); 1, camel milk; 2, cow's milk. (**A**) Comparison of the reactivity of IgG1 antibodies specific for cow's milk diluted 1:3000 (*v*/*v*) towards 5 µg of camel milk and cow's milk (**B**) Comparison of the reactivity of IgG1 antibodies specific for camel milk diluted 1:8000 (*v*/*v*) towards 5 µg of camel milk and cow's milk. (**C**) Cross-reactivity between IgG1 antibodies raised towards cow's milk diluted 1:500 (*v*/*v*) and 40 µg of camel milk proteins. (**D**) Cross-reactivity between IgG1 antibodies raised towards camel milk diluted 1:500 (*v*/*v*) and 40 µg of cow's milk proteins.

4. Discussion

CMA is a major health issue of growing concern, for which reason the World Health Organisation (WHO) has created a guideline for diagnosis and rationale action [4]. Special hypoallergenic infant formulas for CMA infants as well as for infants in risk of developing CMA are available. These infant formulas are based on hydrolysed cow's milk proteins and designated extensively and partially hydrolysed infant formulas, respectively, depending on the degree of hydrolysis and peptide size distribution profile. Additional formulas, based on plants or milk from other mammalians have also been suggested for CMA infants [8]. However, for example, goat and sheep milk cannot be recommended for all CMA infants due to the high protein homology and consequently high cross-reactivity with cow's milk proteins [2,4,12]. One-humped camel—*Camelus dromedaries* (Camelidae family)—has a great evolutionary distance to animals from the Bovidae family [34]. Evolutionary distance directly influences milk protein composition variances, suggesting great differences between camel and cow's milk. Having a different protein composition, camel milk is anticipated to be a suitable alternative to hypoallergenic cow's milk-based infant formulas in the near future. To confirm a role for camel milk in management, primary prevention, and treatment of CMA, a combination of animal and human studies is needed. Using a BN rat model, we have compared immunogenicity, allergenicity, and cross-reactivity of camel and cow's milk proteins.

The present study showed that camel and cow's milk contain similar immunogenicity as well as allergenicity, being able to induce comparable levels of specific IgG1 and IgE antibodies with similar avidity. In addition, the eliciting capacity of the two milk products was shown to be similar. However, evaluation of the specific antibody reactivity towards cross-reactive proteins was low.

Whereas antibody responses raised against caseins were dominated by epitopes of the linear type, antibody responses raised against whey proteins were dominated by conformational epitopes. This is in line with a previous study showing that while caseins primarily raised antibodies towards linear epitopes, BLG and ALA primarily induced antibodies towards conformational epitopes, irrespectively of animals were dosed i.p. or orally [21]. I.p. dosing enables the immune system to recognise proteins in their native, undigested state. These results correlate very well to the structural folding of the proteins within the casein and whey fraction of milk, where caseins possess a flexible unstructured folding [21,35,36], while the predominant proteins within whey, BLG and ALA are globular proteins containing two and four disulphide bonds, respectively [19,21,37].

Camel and cow's milk proteins were in general shown to have a very low cross-reactivity. While approximately only 1 in 30 IgG1 antibodies raised against camel milk could react with cow's milk, only approximately 1 in 50 IgG1 antibodies raised against cow's milk could react with camel milk. The low cross-reactivity was confirmed by inhibitory ELISA where camel milk could only inhibit approximately 35% of the response against cow's milk and cow's milk could only inhibit approximately 50% of the response against camel milk. Similar results were observed for the IgE responses. Low cross-reactivity may reflect differences in the epitope pattern between camel and cow's milk proteins directly correlated with a fairly low protein sequence identities.

The present study demonstrates that camel milk may be a suitable alternative to hypoallergenic infant formulas for CMA infant, as the low cross-reactivity should confer the camel milk low risk of inducing reactions. This is consistent with human studies showing that the introduction of camel milk to children with confirmed CMA, who did not respond to the conventional management, had a positive, rapid and long-lasting effect on their health [20,38]. Other studies have shown that neither camel milk caseins nor whey proteins could inhibit or bind to sera antibodies from patients with confirmed CMA [8,39]. In contrast to camel milk, both goat and sheep milk show a large cross-reactivity to cow's milk [2,14,40], which is also reflected by the high protein identity, causing a similar epitope pattern. Human studies also showed that children with confirmed CMA reacted with goat milk due to IgE antibody cross-reactions [2,15]. In general, goat milk is not recommended for CMA patients without restrictive supervision of specialists [2,14].

The study showed that cow's milk was more efficient in inhibiting binding to antibodies raised against camel milk than camel milk was in inhibiting binding to antibodies raised against cow's milk. Certainly, the lack of BLG, one of the major allergenic proteins in cow's milk [28], may at least partly explain this difference. This indicates that camel milk in general is a more suitable infant formula for CMA infant, than is cow's milk for potential camel milk allergic infants.

The cross-reactivity between camel and cow's milk caseins was found to be less than the cross-reactivity between camel and cow's milk whey proteins, indicating that camel milk would be a more suitable alternative to hypoallergenic infant formulas for casein allergic infants than for whey allergic infants. This corresponds very well to the protein identity within the casein fraction compared to the whey fraction. In addition, immunoblot confirmed that antibodies specific for cow's milk were able to exclusively react with camel milk whey proteins, confirming a predominance of whey proteins cross-reactivity. The reactivity was mostly towards camel milk SA, which is a protein that is rarely detected to independently cause cow's milk allergy, and mostly sensitise together with other milk allergens [41,42].

Small differences were seen between the cross-reactivity accounted for by linear epitopes in comparison to cross-reactivity accounted for by conformational epitopes, where this study indicated that there is a tendency to a lower cross-reactivity between conformational epitopes compared to linear epitopes.

The difference in titre values in each group of immunised rats could reflect weaker antibody binding due to imperfect matching epitopes or be due to a low a low amount of shared epitopes. It can be stressed that the second option is the most likely, as the avidity of the cross-reacting antibodies was equal to the avidity of total population of antibodies.

Overall, it is suggested that approximately 35–40% sequence identity between allergens is adequate to induce IgE cross-reactive binding [43]. However, cross-reactions are unusual below 50% identity and mostly requires more than 70% identity [44]. We can therefore conclude that the low level of cross-reactivity found in the present study is at the expected level for proteins of an evolutionary distance around 60%. A lower cross-reactivity would probably require an even lower sequence homology, which again would require milk from an animal with even larger evolutionary distance to cows. An alternative approach would be to look for milk from animals with a shorter evolutionary distance to humans.

5. Conclusions

This study showed that, although camel and cow's milk display similar immunogenicity and allergenicity, cross-reactivity between their proteins is low. Moreover, selected protein sequence alignments showed lower protein sequence identity between camel and cow's milk proteins in comparison to other mammalian milk proteins such as goat or sheep. With this study, we showed that camel milk is a promising alternative to hypoallergenic cow's milk-based infant formulas. For further evaluation of camel milk and its usefulness as a suitable alternative for hypoallergenic cow's milk-based infant formulas in prevention, treatment and management of CMA, studies including oral animal sensitisation, primary prevention and treatment should be performed. In addition, mechanistic studies, including in vivo analyses of IgE functionality after oral challenge as well as evaluation of cellular changes in the gastrointestinal tract, would be of a great importance.

Author Contributions: N.Z.M. performed all lab work including denaturation of camel and cow's milk, ELISAs and SDS-PAGE electrophoresis and immunoblots. N.Z.M. participated in the lab work and result discussion during the entire study. N.Z.M. did statistical analyses and converted the student report to a paper manuscript. E.B.H. participated in the protein structure analyses as well as immunoblots. E.B.H. was involved in the lab work and result discussion during the entire study. E.B.H. reviewed the manuscript. A.-S.R.B. and A.I.S. participated in SDS-PAGE electrophoresis and immunoblots. A.I.S. especially contributed to the immunoblots optimisation. A.-S.R.B. and A.I.S. reviewed the manuscript. K.L.B. designed the animal experiment and led the animal study. K.L.B. performed the ear swelling test and participated in the denaturation of camel and cow's milk. K.L.B. participated in the lab work and current issues and results discussion during the entire study. K.L.B. reviewed the manuscript.

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Abbreviations

ALA	α-lactalbumin
BLG	β-lactoglobulin
BN	Brown Norway
CMA	Cow's milk allergy
DIG	dioxigenin
ELISA	Enzyme-linked immunosorbent assay
EU	endotoxin units
HRP	horse-radish peroxidase
IC_{50}	half minimum inhibitory concentration
i.p.	intraperitoneally
kDa	kilodalton
KSCN	potassium thiocyanate
LF	actoferrin
LP	lactoperoxidase
MW	molecular weight

NA	not available
OD	optical density
OVA	ovalbumin
PBS	phosphate buffered saline
PBT-T	phosphate buffered saline-tween
PAGE	polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
RT	room temperature
SA	serum albumin
SD	standard deviation
SDS	sodium dodecyl sulphate
TMB	3,3′,5,5′-tetramethylbenzidine
Tris	2-amino-2-(hydroxymethyl)-1,3-propenediol
WHO	World Health Organisation

References

1. Hochwallner, H.; Schulmeister, U.; Swoboda, I.; Spitzauer, S.; Valenta, R. Cow's milk allergy: From allergens to new forms of diagnosis, therapy and prevention. *Methods* **2014**, *66*, 22–33. [CrossRef] [PubMed]
2. Bellioni-Businco, B.; Paganelli, R.; Lucenti, P.; Giampietro, P.G.; Perborn, H.; Businco, L. Allergenicity of goat's milk in children with cow's milk allergy. *J. Allergy Clin. Immunol.* **1999**, *103*, 1191–1194. [CrossRef]
3. Carrard, A.; Rizzuti, D.; Sokollik, C. Update on food allergy. *Eur. J. Allergy Clin. Immunol.* **2015**, *70*, 1511–1520. [CrossRef] [PubMed]
4. Fiocchi, A.; Brozek, J.; Schünemann, H. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. *Pediatric. Allergy Immunol.* **2010**, *21*, 1–125. [CrossRef]
5. El-Agamy, E.I. The challenge of cow milk protein allergy. *Small Rumin. Res.* **2007**, *68*, 64–72. [CrossRef]
6. Many Kids May Not Outgrow Cow's Milk Allergy. Reuters. Available online: https://www.reuters.com/article/us-milk-allergy/many-kids-may-not-outrow-cows-milk-allergy-idUSCOL87231020071218 (accessed on 25 November 2018).
7. Milk and Dairy Allergy. ACAAI. Available online: https://acaai.org/allergies/types-allergies/food-allergy/types-food-allergy/milk-dairy-allergy (accessed on 25 November 2018).
8. El-Agamy, E.I.; Nawar, M.; Shamsia, S.M.; Awad, S.; Haenlein, G.F.W. Are camel milk proteins convenient to the nutrition of cow milk allergic children? *Small Rumin. Res.* **2009**, *82*, 1–6. [CrossRef]
9. Von Berg, A.; Koletzko, S.; Grübl, A.; Filipiak-Pittroff, B.; Wichmann, H.E.; Bauer, C.P.; Reinhardt, D.; Berdel, D.; German Infant Nutritional Intervention Study Group. The effect of hydrolyzed cow's milk formula for allergy prevention in the first year of life: The German Infant Nutritional Intervention Study, a randomized double-blind trial. *J. Allergy Clin. Immunol.* **2003**, *111*, 533–540. [CrossRef] [PubMed]
10. Gateway to Dairy Production and Products. Food Agric. Organ. United Nations. Available online: http://www.fao.org/dairy-production-products/production/dairy-animals/en/ (accessed on 1 January 2018).
11. Järvinen, K.-M.; Chatchatee, P.; Bardina, L.; Beyer, K.; Sampson, H.A. IgE and IgG binding epitopes on α-lactalbumin and β-lactoglobulin in cow's milk allergy. *Int. Arch. Allergy Immunol.* **2001**, *126*, 111–118. [CrossRef]
12. Dean, T.P.; Adler, B.R.; Ruge, F. In vitro allergenicity of cow's milk substitutes. *Clin. Exp. Allergy* **1993**, *23*, 205–210. [CrossRef]
13. Restani, P.; Gaiaschi, A.; Plebani, A.; Beretta, B.; Cavagni, G.; Fiocchi, A.; Poiesi, C.; Velona, A.; Ugazio, G.; Galli, C.L. Cross-reactivity between milk proteins from different animal species. *Clin. Exp. Allergy* **1999**, *29*, 997–1004. [CrossRef] [PubMed]
14. Ehlayel, M.; Bener, A.; Abu Hazeima, K.; Al-Mesaifri, F. Camel Milk Is a Safer Choice than Goat Milk for Feeding Children with Cow Milk Allergy. *ISRN Allergy* **2011**, *2011*, 1–5. [CrossRef] [PubMed]
15. Spuergin, P.; Walter, M.; Schiltz, E.; Deichmann, K.; Forster, J.; Mueller, H. Allergenicity of α-caseins from cow, sheep, and goat. *Allergy* **1997**, *52*, 293–298. [CrossRef] [PubMed]

16. Fiocchi, A.; Dahda, L.; Dupont, C.; Campoy, C.; Fierro, V.; Nieto, A. Cow's milk allergy: Towards an update of DRACMA guidelines. *World Allergy Organ. J.* **2016**, *9*, 1–11. [CrossRef] [PubMed]
17. Lonnerdal, B. Nutritional aspects of soy formula. *Acta Pediatr Suppl.* **1994**, *83*, 105–108. [CrossRef]
18. Hinz, K.; O'Connor, P.M.; Huppertz, T.; Ross, R.P.; Kelly, A.L. Comparison of the principal proteins in bovine, caprine, buffalo, equine and camel milk. *J. Dairy Res.* **2012**, *79*, 185–191. [CrossRef] [PubMed]
19. Hailu, Y.; Hansen, E.B.; Seifu, E.; Eshetu, M.; Ipsen, R.; Kappeler, S. Functional and technological properties of camel milk proteins: A review. *J. Dairy Res.* **2016**, *83*, 422–429. [CrossRef] [PubMed]
20. Navarrete-Rodríguez, E.M.; Ríos-Villalobos, L.A.; Alcocer-Arreguín, C.R.; Del-Rio-Navarro, B.E.; Del Rio-Chivardi, J.M.; Saucedo-Ramírez, O.J.; Sienra-Monge, J.J.L.; Frias, R.V. Cross-over clinical trial for evaluating the safety of camel's milk intake in patients who are allergic to cow's milk protein. *Allergol. Immunopathol.* **2017**, *4*, 149–154. [CrossRef]
21. Nagy, P.; Thomas, S.; Marko, O.; Juhasz, J. Milk production, raw milk quality and fertility of Dromedary camels (Camelus Dromedarius) under intensive management. *Acta Weterinaria Hungarica.* **2013**, *61*, 71–84. [CrossRef]
22. Spencer, P.B.S.; Woolnough, A.P. Assessment and genetic characterisation of Australian camels using microsatellite polymorphisms. *Live Stock Sci.* **2010**, *129*, 241–245. [CrossRef]
23. Nutrition. Available online: https://desertfarms.co.uk/pages/nutrition (accessed on 23 November 2018).
24. Fresh and Frozen Camel Milk. Available online: https://www.oasismilk.com/en/camel-milk/ (accessed on 23 November 2018).
25. Wolkaro, T.; Yusuf Kurtu, M.; Eshetu, M.; Ketema, M. Analysis of camel milk value chain in the pastoral areas of eastern Ethiopia. *J. Camelid Sci.* **2017**, *10*, 1–16.
26. Sawaya, W.N.; Khalil, J.K.; Al-Shalhat, A.; Al-Mohammad, H. Chemical composition and nutritional quality of camel milk. *J. Food Sci.* **1984**, *49*, 744–747. [CrossRef]
27. Madsen, J.; Kroghsbo, S.; Madsen, C.; Pozdnyakova, I.; Barkholt, V.; Bøgh, K.L. The impact of structural integrity and route of administration on the antibody specificity against three cow's milk allergens—A study in Brown Norway rats. *Clin. Transl. Allergy* **2014**, *4*, 25. [CrossRef] [PubMed]
28. Bøgh, K.L.; Barkholt, V.; Madsen, C.B. The sensitising capacity of intact β-lactoglobulin is reduced by Co-administration with digested β-lactoglobulin. *Int. Arch. Allergy Immunol.* **2013**, *161*, 21–36. [CrossRef] [PubMed]
29. Bertino, E.; Gastaldi, D.; Monti, G.; Baro, C.; Fortunato, D.; Garoffo, L.P. Detailed proteomic analysis on DM: Insight into its hypoallergenicity. *Front. Biosci.* **2010**, *2*, 526–536. [CrossRef]
30. Rijnkels, M.; Elnitski, L.; Miller, W.; Rosen, J.M. Multispecies comparative analysis of a mammalian-specific genomic domain encoding secretory proteins. *Genomics* **2003**, *82*, 417–432. [CrossRef]
31. Omar, A.; Harbourne, N.; Oruna-Concha, M.J. Quantification of major camel milk proteins by capillary electrophoresis. *Int. Dairy J.* **2016**, *58*, 31–35. [CrossRef]
32. Broekman, H.C.H.; Eiwegger, T.; Upton, J.; Bøgh, K.L. IgE–the main player of food allergy. *Drug Discov. Today Dis. Model.* **2015**, *17–18*, 37–44. [CrossRef]
33. Levieux, D.; Levieux, A.; El-Hatmi, H.; Rigaudière, J.P. Immunochemical quantification of heat denaturation of camel (Camelus dromedarius) whey proteins. *J. Dairy Res.* **2006**, *73*, 1–9. [CrossRef]
34. Youcef, N.; Saidi, D.; Mezemaze, F.; Kaddouri, H.; Negaoui, H.; Chekroun, A. Cross Reactivity between Dromedary Whey Proteins and IgG Anti Bovine α-Lactalbumin and Anti Bovine β-Lactoglobulin. *Am. J. Appl. Sci.* **2009**, *6*, 1448–1452. [CrossRef]
35. O'Mahony, J.A.; Fox, P.F. Milk Proteins: Introduction and Historical Aspects. *Adv. Dairy Chem.* **2013**, *1*, 43–85. [CrossRef]
36. McMahon, D.J.; Oommen, B.S. Supramolecular Structure of the Casein Micelle. *J. Dairy Sci. Elsevier* **2008**, *91*, 1709–1721. [CrossRef] [PubMed]
37. Beg, O.U.; Bahr-Lindstrom, H.; Zaidi, Z.H.; Jornvall, H. The primary structure of α-lactalbumin from camel milk. *Eur. J. Biochem.* **1985**, *147*, 233–239. [CrossRef] [PubMed]
38. Shabo, Y.; Barzel, R.; Margoulis, M.; Yagil, R. Camel milk for food allergies in children. *Isr. Med. Assoc. J.* **2005**, *7*, 796–798. [PubMed]
39. Restani, P.; Beretta, B.; Fiocchi, A.; Ballabio, C.; Galli, C.L. Cross-reactivity between mammalian proteins. *Ann. Allergy Asthma Immunol.* **2002**, *89*, 11–15. [CrossRef]

40. Wolpe, L.Z.; Silverstone, P.C. A series of substitutes for milk in the treatment of allergies. *J. Pediatr.* **1942**, *21*, 635–658. [CrossRef]
41. Järvinen, K.M.; Chatchatee, P. Mammalian milk allergy: Clinical suspicion, cross-reactivities and diagnosis. *Curr. Opin. Allergy Clin. Immunol.* **2009**, *9*, 251–258. [CrossRef]
42. Natale, M.; Bisson, C.; Monti, G.; Peltran, A.; Garoffo, L.P.; Valentini, S.; Fabris, C.; Bertino, E.; Coscia, A.; Conti, A. Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Mol. Nutr. Food Res.* **2004**, *48*, 363–369. [CrossRef]
43. Kroghsbo, S.; Bøgh, K.L.; Rigby, N.M.; Mills, E.N.C.; Rogers, A.; Madsen, C.B. Sensitization with 7S globulins from peanut, hazelnut, soy or pea induces ige with different biological activities which are modified by soy tolerance. *Int. Arch. Allergy Immunol.* **2011**, *155*, 212–224. [CrossRef]
44. Aalberse, R.C. Structural biology of allergens. *J. Allergy Clin. Immunol.* **2000**, *106*, 228–238. [CrossRef]

4 Manuscript III

Processing induces distinct modifications of cow's and camel milk proteins

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Processing induces distinct modifications of cow's and camel milk proteins

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Abstract

Enzyme hydrolysis is a processing method commonly used for reducing protein allergenicity in the production of hypoallergenic infant formulas, whereas heat treatment is mainly used for microbial safety assurance in the dairy industry. Infant formulas are primarily based on cow's milk proteins, but recently milk proteins from other mammalian species, including camel milk proteins, have gained an interest for their usability in infant formula production. In this study, we showed that enzyme hydrolysis and heat treatment affected cow's and camel milk proteins differently, which we hypothesise is primarily due to the differences in composition of their proteins and other milk components. For both processing methods, lack of β-lactoglobulin in camel milk played an important role in causing differences in proteins modifications between cow's and camel milk. When using heat treatment and enzyme hydrolysis in the production of infant formula, in order to obtain similar degree of proteins allergenicity reduction in cow's and camel milk, specific processing parameters should be applied individually to those two types of milk.

Keywords: milk processing, enzyme hydrolysis, heat treatment, protein modification

1. Introduction

Cow's milk is the most common mammalian milk used in the dairy industry, constituting 81% of the total milk production worldwide [16]. Thus, cow's milk is also the most common source of protein for infant formula production [40].

Enzyme hydrolysis is a frequently utilised processing technology in the infant formula industry, used to reduce the allergenicity of cow's milk proteins and hence for production of hypoallergenic infant formulas [28,41,42]. Enzyme hydrolysis leads to degradation of proteins, thus to peptides formation, where the extent of proteolysis depends on the protein:enzyme ratio, enzyme specificity, numbers of enzymes as well as process parameters such as time, temperature and pH [43]. The susceptibility to hydrolysis differs between different milk proteins, where it has been shown that the whey proteins: α-lactalbumin (ALA), serum albumin (SA), ferritin as well as caseins are more susceptible to hydrolysis than ß-lactoglobulin (BLG) [43,44].

Heat treatment, on the other hand, is a frequently utilised processing technology in dairy industry primarily used for microbial safety assurance [45]. Yet, it has recently gained a research interest for its applicability as a tool for altering protein structure and thus its potential in reducing allergenicity [7,46]. Heat treatment can lead to protein denaturation, aggregation and glycation [47,48], where the extent of changes in protein structure depends on the temperature and time applied, the initial milk protein structures as well as the availability of other components such as fat and sugar [49,50]. For example, it is known that caseins are lacking secondary, tertiary and quaternary structures that are normally destroyed under heat treatment, making them more heat stable than globular proteins from the whey fraction [28]. During high processing temperatures, caseins form aggregates together with denatured whey proteins, especially with interaction between κ-casein and BLG thiol groups [51–53]. In addition, heat treatment results in Maillard reaction which is initiated by the interaction of a free amine group and a reducing sugar. In the late stage of Maillard reaction cross-linked compounds and potentially their aggregate formation can occur. Another mechanism of protein cross-linking is the sugar-independent reactions between dehydoalanine (DHA) and lysine or cysteine resulting in lysinoalanine (LAL) and lanthionine (LAN), respectively [46,54–56].

Recently, camel milk has gained an interest as a source of protein in the dairy industry especially in the production of infant formulas [57]. Cow's and camel milk contain comparable amounts of water, protein, fat, lactose and ash [22]. Moreover, camel milk lacks BLG, the most abundant protein found in cow's milk whey fraction [23,58], but instead ALA is the most abundant protein in the whey fraction of camel milk [59]. In general, counterpart proteins in cow's and camel milk have low sequence identity ranging between 47 and 81% [21]. The casein fraction of camel milk contains more β-casein and less κ-casein than the cow's milk casein fraction [22]. Caseins from camel milk also form bigger micelles when comparing with cow's milk, making them less prone

to precipitate and thus to form aggregation [16]. On the other hand, κ-casein and BLG in cow's milk are known to play an important role in aggregates upon heat treatment formation [52,60]. Therefore it could be hypothesised that lack of BLG and less k-casein in camel milk could make it less prone for aggregates upon heat treatment formation. Further, denaturation of ALA at 90 ˚C in camel milk was reported to be lower than denaturation of ALA and BLG in cow's milk [17]. Higher level of denaturation of ALA and BLG in cow's milk leads to their enhanced interactions with casein micelles [17,51].

Collectively, processing parameters and milk composition have a direct effect on the resulting functional properties of milk products [22]. Hence, proteins from cow's and camel milk will behave differently under heat treatment and enzyme hydrolysis, processing technologies that are important in the infant formula industry to produce hypoallergenic infant formulas. While cow's milk proteins and the effect of processing are well studied [48,61], knowledge on camel milk proteins and how processing influences their properties, and especially how they differs with cow's milk is scarce. In this study, we investigated changes in the physicochemical properties of cow's and camel milk proteins after heat treatment and enzyme hydrolysis, respectively. With this study, we provide new knowledge that may be used for future infant formula production based on camel milk proteins.

2. Materials and methods

2.1. Milk products

Full fat cow's milk powder from MlekPol (Grajewo, Poland) was purchased in a local Polish shop with the composition declared on the package as g/100g powder: fat 27 g, carbohydrate 38 g, protein 26 g. Full fat camel milk powder was kindly provided by Ausnutria Dairy (China) Co., Ltd, (Changsha, Hunan, China) with the composition declared on the package as g/100g powder: fat 22.9 g, carbohydrate 39.1 g, protein 24.4 g.

2.1.1. Preparation of milk products

A total of six different milk products were prepared as follows: intact full fat cow's milk, heat treated (HT) full fat cow's milk, enzyme hydrolysed (EH) full fat cow's milk, intact full fat camel milk, HT full fat camel milk, and EH full fat camel milk as described below.

2.1.1.1. Intact full fat cow's and camel milk

Intact full fat cow's and camel milk powders were dissolved in sterile phosphate buffer saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) to obtain 50 mg/mL of protein as an initial concentration for further experiments and shored at -20 ˚C.

2.1.1.2. HT full fat cow's and camel milk

To prepare HT full fat cow's and camel milk, intact full fat cow's and camel milk with protein concentrations of 50 mg/mL were used, and heat treatment was performed at 121 ˚C for 1 h. HT cow's and camel milk were stored at -20 ˚C until further analyses.

2.1.1.3. EH full fat cow's and camel milk

To prepare EH full fat cow's and camel milk, Pancreatic Trypsin Novo 6.0 S, Type Salt Free (Trp/Chtrp) (trypsin activity 1400 units mg^{-1} protein, chymotrypsin activity: 79 units mg^{-1} protein, Novozymes, Bagsværd, Denmark) and Alcalase 2.4 L FG (Alc) (activity: 2590 units mg^{-1} protein, Novozymes) were used. First, the pH was adjusted to 7.5 for optimal enzymes activities. Next, intact full fat cow's and camel milk were heated at 90 ˚C for 15 min for protein unfolding and subsequently, the temperature was adjusted to 55 ˚C for optimal enzyme activities. Based on a pilot study, optimal conditions for comparable partial hydrolysis of intact full fat cow's and camel milk were applied, where 0.5% Trp/Chtrp (w/w) and 0.05% Alc (v/v) were added to intact full fat cow's milk and cow's milk proteins were hydrolysed for 3 h, while 0.5% Trp/Chtrp (w/w) and 0.1% Alc (v/v) were added to intact full fat camel milk and camel milk proteins were hydrolysed for 7 h. Finally, products were heated at 90 ˚C for 15 min for enzymes deactivation, cooled down to room temperature (RT) and stored at -20 ˚C until further analyses.

2.1.2. Defatting of milk products

In order to obtain milk products without fat particles, a two-step chemical defatting was performed. Solutions of 10 mL of intact full fat cow's milk, HT full fat cow's milk, EH full fat cow's milk, intact full fat camel milk, HT full fat camel milk and EH full fat camel milk with protein concentrations of 50 mg/mL were prepared and samples were freeze dried at -54 ˚C, 0.3 mbar, using Lyovapor™ L-200 (Buchi, Flawil, Switzerland) for 72 h. The freeze-dried milk powders were weighted and stored at -20 ˚C until further defatting procedure. Subsequently, absolute EtOH (1:10 w/v) (20821310, VWR, Lutterworth, UK) was added to each of six milk powders and stirred for 2 h at RT under the fume hood using a magnetic stirrer. Further, each milk powder was recovered by filtration on Whatman™ filter papers grade 1 (1001-125, Merck, Dermstadt, Germany) on a glass funnel. Milk powders were left to dry overnight under the fume hood. Next, powders were carefully moved to a glass beaker and weighted. The defatting procedure was repeated as described above using n-hexane (1:5 w/v) (1043672511, Merck). Finally, milk products without fat particles were re-dissolved in sterile PBS and stored at -20 ˚C until further use. Defatted milk samples were further used for all analyses preformed in this study.

2.2. Product analyses

In order to evaluate how heat treatment and enzyme hydrolysis influenced the physicochemical properties of cow's and camel milk proteins, the following analyses were performed.

2.2.1. LC-MS/MS

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed in order to evaluate intact cow's and camel milk proteins composition and their relative amounts. The sample preparation and LC-MS/MS analysis was performed at the Technical University of Denmark (DTU) Proteomics Core. The raw files obtained after LC-MS/MS were analysed using MaxQuant version 1.6.10.43 [62]. A custom database composed of the major milk protein sequences from camel and cow was used. The database consists of sequences with the accession numbers (https://www.uniprot.org) listed in table S1 (Supplementary material). The digestion mode was set to "specific trypsin/P" as samples treated with trypsin before the run. Variable modifications were set to: Oxidation (M); Acetyl (Protein N-term); and Phospho (ST). Fixed modification to: Carbamidomethyl (C). Minimum and maximum peptide lengths were set to 2 and 40 amino acid (aa) respectively, and the maximum number of modifications per peptide was set to 8. Label-free quantitation LFQ and iBAQ was enabled.

2.2.2. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described [21] with minor changes. Briefly, 10 µL of Precision Plus Protein™ Dual Xtra Standard (1610377, Bio-Rad, Hercules, CA, US) as well as 15 µg of protein in intact cow's milk, HT cow's milk, intact camel milk, HT camel milk and 30 µg of protein in EH cow's milk and EH camel milk in 2x Laemmli buffer (1610737, Bio-Rad) and 50 mM dithiothreitol (DTT, D0632, Merck) were loaded onto a 4-20% precast polyacrylamide gel (4568094, Bio-Rad). Samples were run on the gel for 30 min at 200 V with constant current at RT. After the run, the gel was stained for 3 h with Bio Safe™ Coomassie (161-0786, Bio-Rad) and photographed as described before [21].

2.2.3. O-phthalaldehyde assay

O-phthalaldehyde (OPA) assay was performed in order to evaluate the degree of hydrolysis (DH) of EH cow's and camel milk proteins. The protocol used was as described by Hussein et al. [63] with some modifications. Briefly, OPA reagent was prepared with three solutions. Solution 1 consisted of 3.81 g sodium tetraborate (221732, Merck) and 100 mg SDS (L3771, Merck) dissolved in 75 mL MilliQ water, solution 2 consisted of 80 mg OPA (P0657, Merck) dissolved in 2 mL of 96% EtOH (20923290, VWR) and solution 3 consisted of 88 mg DTT (D0632, Merck) dissolved in 50 mL MilliQ water. Solution 1, 2 and 3 were prepared individually and mixed together before each OPA assay. Two-fold dilution of 10 mM serine (S4500, Merck) was used for standard curve preparation and DH calculation. DH was calculated based on the equation: DH= (Δmg of serine equivalent/mg protein)*100%, where "Δmg of serine equivalent" is the serine equivalents after hydrolysis subtracted with the serine equivalents of unhydrolysed product), mg protein was 1 mg of protein

for each product. Intact cow's milk, EH cow's milk, intact camel milk and EH camel milk (36 µL of 1 mg/mL) were added to a 96-well microplate (269620, Nunc, Roskilde, Denmark). Subsequently, 270 µL of freshly mixed OPA reagent was added to each well, incubated for 2 min and the absorbance was measured at 340 nm using EnSpire® Multimode Plate Reader (PerkinElmer®, Walthman, MA, US). All samples were run in duplicates with PBS as blank.

2.2.4. LC-MS/MS triple Q based Multiple Reaction Monitoring (MRM)

To evaluate Maillard reaction markers after heat treatment of cow's and camel milk, absolute quantification of furosine, N-ε-(carboxyethyl)lysine (CEL), bN-ε-(carboxymethyl)lysine (CML), LAL, LAN and lysine was conducted as previously described using LC-MS/MS triple Q based Multiple Reaction Monitoring (MRM) [54]. Briefly, 100 µL (1 mg/mL) of intact cow's milk, HT cow's milk, camel milk and HT camel milk were mixed with 200 µL of 10 M HCl. Nitrogen was flown into the samples for oxygen depletion before the sample was incubated for 24 h at 110 °C for acid hydrolysis. The hydrolysed samples were added 700 µL of water and centrifuged at 14,000 *g* for 15 min. Supernatants were collected and dried by vacuum centrifugation (SP Scientific, United States), and subsequently re-dissolved in 400 µL of water and filtered (Whatman filters, 0.2 µM) before loading onto an LC Infinity 1260 system coupled to a 6460 Triple Quad mass spectrometer (Agilent Technologies, Waldbronn, DE), which operated in MRM acquisition mode. Quantification was calculated based on the ratio of the analytes and their internal standards and compared to a calibration curve of known concentrations.

2.2.5. Gel Permeation Chromatography

Milk products were analysed for protein separation profile using gel permeation chromatography (GPC) under either physiological conditions, with 6 M urea (29700, Thermo Fisher Scientific) or with 6 M urea (29700, Thermo Fisher Scientific) and 5 mM DTT (D9779, Merck) at RT on a Superdex 200 Increase 10/300 GL column (28990944, Cytiva, Marlborough, MA, US) coupled to a ÄKTA pure 25 system (Cytiva, Marlborough, MA, US). Intact cow's milk (50 µL of 5 mg/mL), HT cow's milk (50 µL of 10 mg/mL), EH cow's milk (50 µL of 5 mg/mL), intact camel milk (50 µL of 5 mg/mL), HT camel milk (50 µL of 10 mg/mL) and EH camel milk (50 µL of 5 mg/mL) were injected onto the column after being filtered through a 0.45 µm filter. Milk samples were eluted at 0.75 mL/min with either PBS buffer as physiological conditions, PBS buffer with addition of 6 M urea or PBS buffer with 6 M urea and 5 mM DTT. Elution of the samples was monitored at 215 and 280 nm. A mixture of standard proteins consisting of 0.3 mg/mL ferritin (440 kDa, F4503, Merck), 1 mg/ml ovatransferrin (79 kDa, C0880, Merck), 1 mg/ml carbonic anhydrase (29 kDa, C3934, Merck), 1 mg/ml cytochrome C (14 kDa, C2506, Merck), 1 mg/ml apotinin (6 kDa, A1153, Merck) and 0.5 mg/ml vitamin B12 (1.3 kDa, V2876, Merck) was filtered through 0.2 µm filter and applied to the column under physiological conditions in order to calibrate for molecular weight (MW) determination.

Data was generated though accessing research infrastructure at DTU, National Food Institute, including FOODHAY (Food and Health Open Innovation Laboratory, Danish Roadmap for Research Infrastructure).

3. Results and discussion

Intact cow's milk, HT cow's milk, EH cow's milk, intact camel milk, HT camel milk and EH camel milk samples were prepared and analysed in order to evaluate physicochemical changes of the proteins after heat treatment and enzyme hydrolysis.

3.1. Protein composition

LC-MS/MS was run to analyse the peptide composition in intact cow's and camel milk. From the analysis performed, we could estimate the relative protein composition in tryptic digest cow's and camel milk. Caseins were shown to be the main proteins in both cow's and camel milk, where β-casein was found to be the most abundant casein in both cow's and camel milk, though it was more abundant in camel milk than in cow's milk (Table 1). κ-casein was on the other hand more abundant in cow's milk (Table 1). While BLG was the most abundant whey protein in cow's milk, the results confirmed the lack of BLG in camel milk where the main whey proteins were ALA and glycosylation-dependent cell adhesion molecule-1 (GLYCAM-1) (Table 1). SA and lactoferrin (LF) were the least abundant proteins in cow's and camel milk, and while whey acidic protein (WAP) was identified in camel milk, it was not present in cow's milk (Table 1).

Table 1: Cow's and camel milk protein composition. Results shown as % of protein in relation to the total amount of proteins. **ALA,** α-lactalbumin; **BLG,** β-lactoglobulin; **cas,** casein; **GLYCAM-1,** Glycosylation-dependent cell adhesion molecule-1; **LF,** lactoferrin; **SA,** serum albumin; **WAP,** whey acidic protein. **NA,** not identified.

Protein / Product	αs1-cas	αs2-cas	β-cas	κ-cas	BLG	ALA	SA	GLYCAM-1	WAP	LF
Cow's milk	22.7	13.5	26.8	9.4	13.0	10.8	0.6	1.44	NA	0.3
Camel milk	17.8	7.9	32.8	7.8	NA	16.0	1.4	13.8	1.0	0.2

3.2. Protein profile

SDS-PAGE was run to demonstrate differences in protein profile of the intact cow's and camel milk as well as their HT and EH versions. Caseins appeared as an intensive band between 25 and 37 kilodalton (kDa) in both intact cow's and camel milk (Lane 1 and 4, Figure 1), which was as expected based on the results in Table 1 as they were shown to be the main proteins in both cow's and camel milk. In intact cow's milk (Lane 1, Figure 1), a band corresponding to BLG was visible between 15 and 20 kDa, while no similar band in intact camel milk was detected (Lane 4, Figure 1) as camel milk lacks BLG (Table 1). Both intact cow's milk and intact camel milk had a band around 15 kDa that corresponds to ALA (Lane 1 and 4) as well as a band just below 75 kDa that corresponds to SA (Lane 1 and 4). A band above 75 kDa was only visible in intact camel milk,

probably representing LF (Lane 4, Figure 1). In addition a weak band around 20 kDa in intact camel milk (Lane 4) might represent glycam-1 as from LC-MS/MS it was concluded that it is a whey protein much more abundant in intact camel milk when comparing with intact cow's milk. Both intact cow's and camel milk contained immunoglobulins (Igs) between 150 and 250 kDa (Lane 1 and 4). Whereas intact cow's and camel milk presented as clear individual bans in the gel, HT cow's and camel milk presented as undefined bands as a result of heat treatment causing protein aggregation in both products (Lane 2 and 5). Protein aggregates presented as a band on the top of both wells in Lane 2 and 5 indicating lack of the mobility into the gel. EH cow's and camel milk (Lane 3 and 6, Figure 1) showed no intact proteins, indicating their complete hydrolysis (Lane 3 and 6, Figure 1). In both Lane 3 and 6, only a smear of bands below 10 kDa was visible indicating the presence of smaller peptides.

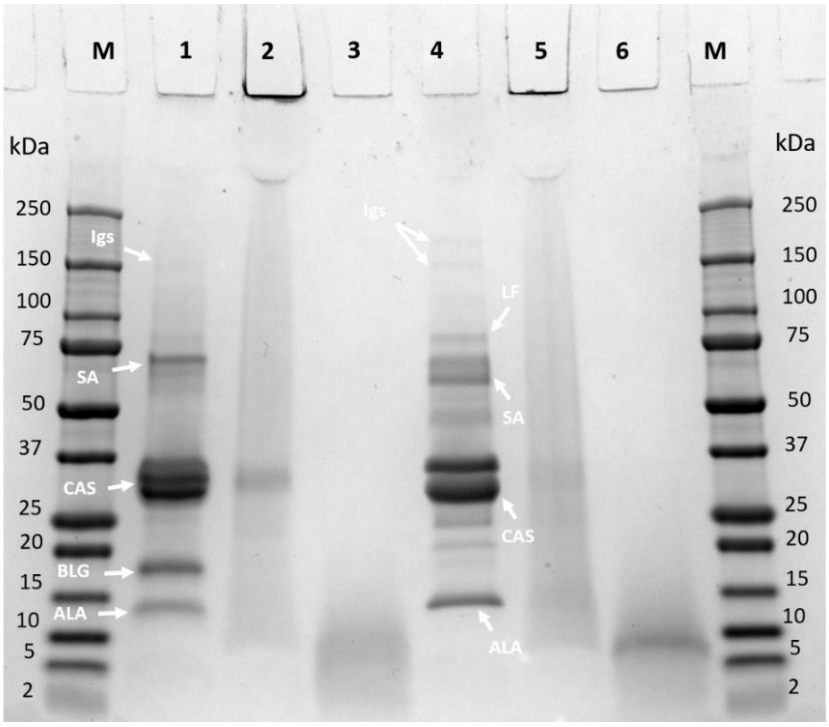

Figure 1. SDS-PAGE. Gel electrophoresis under reducing conditions with intact, heat treated (HT) and enzyme hydrolysed (EH) cow's and camel milk. **M** protein molecular weight standard, **1** cow's milk, **2** HT Cow's milk, **3** EH Cow's milk, **4** Camel milk, **5** HT Camel milk, **6** EH Camel milk. **ALA**, α-lactalbumin; **BLG**, ß-lactoglobulin; **CAS**, caseins; **SA**, serum albumin; **Igs**, immunoglobulins; **kDa**, kilodalton; **LF,** lactoferrin.

3.3. Degree of hydrolysis

To determine DH of EH cow's and camel milk after enzyme hydrolysis with Trp/Chtrp and Alc, the concentration of N-terminal nitrogen was determined by the OPA assay using serine as standard. DH was calculated from the increase in N-termini relative to total protein. DH was estimated to be approx. 12% for EH cow's milk and approx. 15% for EH camel milk. The slightly higher DH of

EH camel milk compared to EH cow's milk could be explained by the lack of BLG, in which is the main whey protein in cow's milk and is known to be more resistant to proteolysis than other milk proteins [21,43,44]. While Alc hydrolyses peptide chains at the carboxyl side of most of the aa [64], Trp hydrolyses peptide chains at the carboxyl side of lysine and arginine and Chtrp hydrolyses peptide chains at the carboxyl side of aromatic aa [65]. Differences in DH observed between EH cow's and camel milk after enzyme hydrolysis with Alc and Trp/Chtrp could be explained by the differences in aa profile of cow's and camel milk proteins. Enzyme hydrolysis may be used for infant formula based on camel milk proteins to improve their utility especially in production of hypoallergenic infant formula by even further reduction of their allergenicity and cross-reactivity with cow's milk proteins. When aiming for the comparable DH of camel milk proteins to the DH of cow's milk proteins in hypoallergenic infant formula, we hypothesise that enzyme hydrolysis parameters should be individually selected for proteins hydrolysis in camel milk.

3.4. Maillard reaction markers and cross-links

LC-MS/MS triple Q based MRM was used in order to evaluate the degree of Maillard reaction and DHA-mediated protein cross-linking occurring after heat treatment of cow's and camel milk. The level of sugar independent cross-links such as LAL and LAN, and the early stage Maillard reaction marker furosine, as well as the advanced glycation end products (AGE) such as CEL and CML were determined in intact as well as HT cow's and camel milk. In intact cow's and camel milk only furosine was detected in sufficient levels to be quantified, which was probably created during milk powder production (Table 2), while CEL, CML, LAN and LAL were under the limit of quantification. HT cow's and camel milk had comparable level of furosine, CML, and LAN but the level of LAL and CEL differed. There was more than twice the level of LAL in HT cow's milk (12.15 ± 0.30 mg/g) compared to HT camel milk (5.50 ± 0.02 mg/g) and almost two times more CEL in HT cow's milk (0.24 ± 0.03 mg/g) than in HT camel milk (0.13 ± 0.00 mg/g). More LAL in HT cow's milk could be explained by a higher content of phosphoserine in cow's milk compared to camel milk. Phosphorylated serine residues are known to be more reactive as precursors for the DHA formation in the reaction [55]. Caseins contain phosphoserine clusters allowing the caseins to keep insoluble calcium phosphate in colloidal form. β-casein have 5 phosphoserines whereas the α-caseins have 8-13 phosphorylated residues [66]. Camel milk has a relative higher content of β-casein and relatively lower content of α-caseins compared to cow's milk as shown in Table 1, and the content of phosphoserine will therefore expectedly be lower in camel milk. Free lysine content was slightly higher in intact camel milk when comparing with intact cow's milk and for both HT camel and cow's milk was reduced (Table 2).

Table 2: Maillard reaction products and cross-links. Results are shown as mg/g of protein. **CEL**, N-ε-(carboxyethyl)lysine; **CML**, bN-ε-(carboxymethyl)lysine; **LAN**, lanthionine; **LAL**, lysinoalanine; **LOQ**, limit of quantification.

Product	CEL (mg/g)	CML (mg/g)	LAN (mg/g)	LAL (mg/g)	Furosine (mg/g)	free lysine (mg/g)
Intact cow's milk	<LOQ	<LOQ	<LOQ	<LOQ	2.46 ± 0.01	72.90 ± 1.35
HT cow's milk	0.24 ± 0.03	0.62 ± 0.01	0.64 ± 0.04	12.15 ± 0.30	6.79 ± 0.20	61.33 ± 0.47
Intact camel milk	<LOQ	<LOQ	<LOQ	<LOQ	1.99 ± 0.04	82.81 ± 1.04
HT camel milk	0.13 ± 0.00	0.56 ± 0.00	0.50 ± 0.00	5.50 ± 0.02	6.26 ± 0.22	57.85 ± 0.39

3.5. Molecular size distribution and aggregation profile

To evaluate proteins molecular size distribution and their aggregation profile in intact cow's milk, HT cow's milk, EH cow's milk, intact camel milk, HT camel milk and EH camel milk, were analysed by GPC under physiological conditions. Figure 2A displays chromatograms of intact cow's milk, HT cow's milk and EH cow's milk at 215 nm corresponding to the absorbance of peptide bonds. Proteins in intact cow's milk were eluted as large complexes represented as a peak above 440 kDa, followed by several not fully separated peaks between 440 and 14 kDa, and finally several peaks below 14 kDa. Proteins in HT cow's milk showed a different pattern of elution (Figure 2A) except for the large protein complexes represented by a peak with MW above 440 kDa that remained after heat treatment of cow's milk. For proteins between 79 and 6 kDa their separation was reduced, indicating heavily aggregated protein complexes after the heat treatment process. In EH cow's milk, only small proteins and peptide complexes were detected, running as one big peak around 6 kDa with a shoulder peak at around 14 kDa (Figure 2A).

Figure 2B displays chromatograms of intact camel milk, HT camel milk and EH camel milk at 215 nm. As for intact cow's milk (Figure 2A), proteins in intact camel milk were eluted as a complexes, starting with an elution of large protein complexes above 440 kDa, several not fully separated peaks between 440 and 14 kDa and several not fully separated peaks below 14 kDa. Proteins in HT camel milk showed quite different pattern of proteins elution when comparing with camel milk as shown in Figure 2B. First of all, peak of aggregated protein complexes above 440 kDa was smaller after heat treatment of camel milk indicating that probably it was formed by proteins that denatured after heat treatment and partially left this big complex of aggregated proteins. This could also be caused by the fact that β-casein in camel milk is more loosely associated than its counterpart in cow's milk [15,22], and thus could be less prone to form larger complexes under heat treatment. In addition, some peaks between 79 and 6 kDa disappeared after heat treatment but on the other hand, new peaks appeared indicating a formation of new protein complexes upon heat treatment. In comparison to the profile of proteins in HT cow's milk (Figure 2A) that showed more unresolved peaks after heat treatment, it may indicate that more protein

modifications, thus aggregation were created after heat treatment in cow's when comparing with camel milk. This might be because of the presence of BLG unpaired cysteins that under heat treatment above 70 ^{o}C react with casein micelles especially κ-casein on their surface and has catalysing effect on proteins aggregation [51,56,67]. This is in agreement with the results from LC-MS/MS triple Q based MRM analysis that showed higher level of mainly sugar independent aa cross-linking caused by heat treatment in cow's milk when comparing with camel milk. EH camel milk occurred as one big peak around 6 kDa, representing aggregated peptide complexes formed after protein proteolysis during the enzyme hydrolysis process as shown in Figure 2B. When comparing with EH cow's milk (Figure 2A), it did not contain the additional clustered peak at around 14 kDa indicating higher extent of enzyme hydrolysis in camel milk than in cow's milk which is in line with DH of EH cow's and camel milk.

For all six milk products, elution was also monitored at 280 nm, representing the detection of aromatic aa, and provided chromatographs similar to those at 215 nm as shown in Figure S1 (Supplementary material).

From the perspective of the utility of heat treatment to alter allergenicity of proteins to be used in infant formula, it would be important to highlight that depending on the extent of heat treatment, there is a balance between protein denaturation, aggregation and glycation. This will have a direct influence on the protein structure and whether the allergenicity of protein will be decreased or actually increased by exposure of the sites of protein that were not available before heat treatment of by formation of new structures that lead to proteins increased allergenicity. As cow's and camel milk behaved differently under heat treatment, when aiming for similar modifications of their proteins under heat treatment, we hypothesise that even more extended heat treatment should be applied to obtain more aggregation of camel milk proteins.

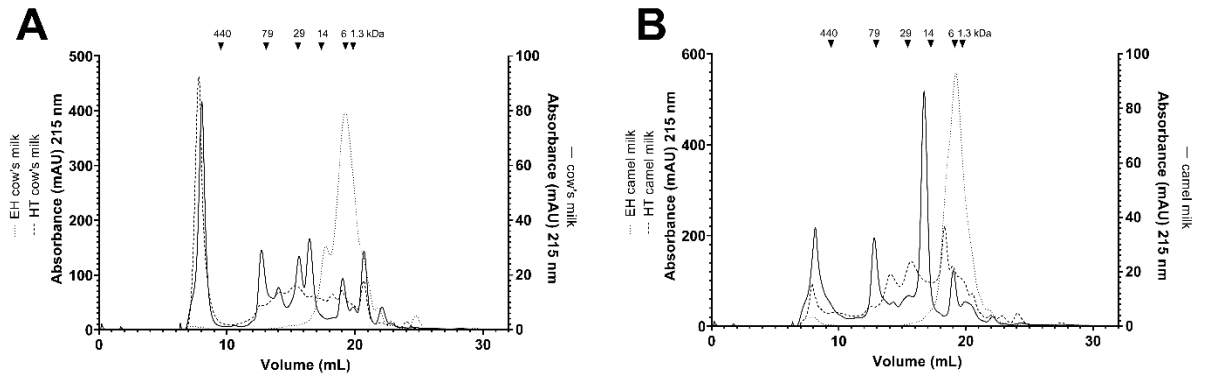

Figure 2. Gel permeation chromatography under physiological conditions at 215 nm. (A) Protein separation profile of —— cow's milk, --- HT cow's milk and ···· EH cow's milk. **(B)** Protein separation profile of —— camel milk, --- HT camel milk and ···· EH camel milk.

In order to evaluate what type of interactions dominated in aggregated protein complexes in cow's milk, HT cow's milk, EH cow's milk, camel milk, HT camel milk and EH camel milk, GPC of each product was analysed under physiological conditions as described above as well as under denaturing conditions in 6 M urea or in 6 M urea containing 5 mM DTT and monitored at 280 nm. Urea disrupts non-covalent bonds such as ionic bonds, hydrogen bonds and Van der Waals bonds, leading to protein denaturation by tertiary structure disruption [68], whereas DTT is responsible for breaking disulphide bonds [69].

Figure 3A and 3B display cow's milk and camel milk chromatograms under physiological or denaturing conditions. In the cow's milk sample, the peak of heavily aggregated proteins above 440 kDa remained after addition of urea whereas the peak was reduced by addition of urea and DTT, indicating that cysteine disulfide bridges are important for these large complexes in cow's milk (Figure 3A). In the camel milk sample, the peak above 440 kDa was reduced with addition of urea alone, indicating that large protein complexes in camel milk were held together with non-covalent interactions rather than disulfide bonds (Figure 3B). For both products, the other peaks shifted towards a faster elution when products were run with urea and urea + DTT indicating denaturation and unfolding of protein structures and further aggregation (Figure 3A and B).

Figure 3C and 3D display HT cow's milk and HT camel milk chromatograms under physiological or denaturing conditions. For both products, no major changes could be seen in the peak pattern after addition of urea or urea containing DTT, indicating that strong interactions in protein complexes formed under heat treatment, making them hard to separate. However, for peaks below 6 kDa, it could be seen that they eluted faster when diluted in urea and urea containing DTT, indicating slight denaturation and unfolding of small peptide complexes and further aggregation.

Figure 3E and 3F display EH cow's milk and EH camel milk chromatograms under physiological or denaturing conditions. For both products, peptides eluted slightly faster when urea or urea containing DTT was present, which might be explained by their denaturation and unfolding by urea and further caused aggregation.

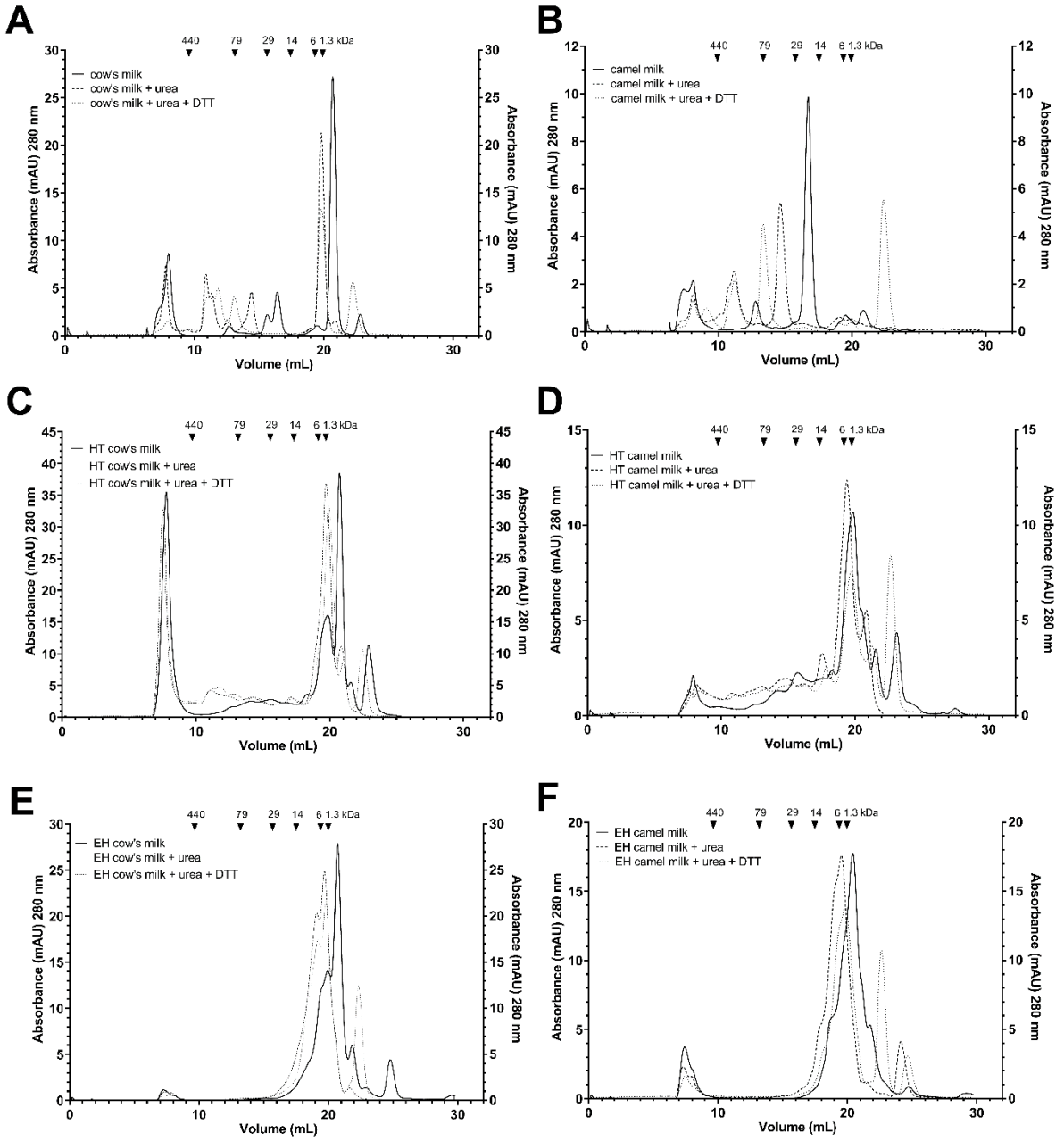

Figure 3. Gel permeation chromatography of milk products under physiological conditions, in urea or in with urea + dithiothreitol (DTT) at 280 nm. (A) Protein separation profile of — cow's milk, --- cow's milk + urea, and ···· cow's milk + urea + DTT. **(B)** Protein separation profile of — camel milk, --- camel milk + urea and ···· camel milk + urea + DTT. **(C)** Protein separation profile of — HT cow's milk, --- HT cow's milk + urea and ···· HT cow's milk + urea + DTT. **(D)** Protein separation profile of — HT camel milk, --- HT camel milk + urea and ···· HT camel milk + urea + DTT. **(E)** Protein separation profile of — EH cow's milk, --- EH cow's milk + urea and ···· EH cow's milk + urea + DTT **(F)** Protein separation profile of — EH camel milk, --- EH camel milk + urea and ···· EH camel milk + urea + DTT.

4. Conclusions

In this paper, we showed that proteins from cow and camel milk behaved differently under heat treatment and enzyme hydrolysis. The differences could be explained by the absence of BLG in camel milk and a large difference in the relative composition of the other milk proteins. When using heat treatment and enzyme hydrolysis in the production of infant formula, in order to obtain similar degree of proteins allergenicity reduction in cow's and camel milk, specific processing parameters should be applied individually to those two types of milk.

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5 Manuscript IV

Sensitising capacity of cow's and camel milk products in a Brown Norway rat model – the impact of processing

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Manuscript in preparation.

Sensitising capacity of cow's and camel milk products in a Brown Norway rat model – the impact of processing

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Abstract

Background: Infant formulas are breast milk substitutes when breastfeeding is impossible or insufficient. They are mainly based on cow's milk proteins or their hydrolysed versions when they are used in the production of infant formula dedicated for cow's milk allergy (CMA) management. Camel milk was shown to be well tolerated in majority of small children with CMA, and there is a hypothesis that enzyme hydrolysis and heat treatment could improve usefulness of camel milk even further.
Methods: The aim of this study was to evaluate how processing such as enzyme hydrolysis and heat treatment influenced immunogenicity, sensitising and cross-reactive capacity of cow's and camel milk proteins. For this purpose Brown Norway rats were immunised i.p. three times with intact cow's milk, heat treated (HT) cow's milk, enzyme hydrolysed (EH) cow's milk, intact camel milk, HT camel milk and EH camel milk. Antibodies responses, immunoblotting as well as in vivo test were used.
Results: Cow's and camel milk showed similar immunogenicity and sensitising capacity. EH cow's and camel milk both showed decreases immumnogenicity and sensitising capacity while they differed between HT cow's and camel milk. In addition, this study showed that there was a low cross-reactivity between cow's and camel milk proteins that was further decreased with processing.
Conclusions: This study showed that heat treatment and enzyme hydrolysis influenced cow's and camel milk immunogenicity, sensitising capacity, reactivity and its specificity in different ways. In addition heat treatment and enzyme hydrolysis showed to further decrease cross-reactivity between cow's and camel milk proteins.

Keywords: cow's milk allergy, heat treatment, enzymatic hydrolysis, immunogenicity, sensitising capacity, cross-reactivity

1. Introduction

Cow's milk allergy (CMA) is known to be the most frequent food allergy in infants and small children, affecting 0.5-3.8% of them worldwide [5,70,71]. Although many small children are outgrowing CMA by the age of three years [72], there is a trend to a later CMA outgrowth or to keep it life-long [73].

Breastfeeding is suggested as a primary choice of infants feeding [74], but if breastfeeding is not possible and an infant suffers from CMA, a use of hypoallergenic infant formula based on enzyme hydrolysed cow's milk proteins is recommended [42,75]. Based on the degree of hydrolysis (DH), there are two types of hydrolysed cow's milk protein based formulas: extensively hydrolysed formula (eHF) and partially hydrolysed formula (pHF) [76]. eHFs are used in CMA management as they mostly consists of peptides <3 kDa [77], thus they have a low risk to elicit allergic symptoms [78,79]. On the other hand, pHFs were until recently recommended for CMA prevention. However current guidelines do not recommend the use of pHFs in CMA prevention [39,80,81], due to the lack of evidences supporting preventive capacity of pHFs in human studies [82–84]. In general, immunogenicity and sensitising capacity of hydrolysed cow's milk proteins have been studied in several animal studies, though with different outcomes caused by differences in their DH [85–91].

Heat treatment is a processing method that has gained scientific interest for its potential to reduce allergenicity of cow's milk proteins [49,92]. Several human studies have shown that baked milk could be tolerated in children with CMA [93–96], which indicated reduced allergenicity of cow's milk proteins after extensive heat treatment. On the other hand, Abbring et al. also in a human study showed that heat treated whey proteins had an increased allergencity compared to unprocessed whey proteins [97]. Results from animal studies evaluating sensitising and eliciting capacity of heat treated cow's milk are also conflicting. For example Graversen et al. showed that while heat treated whey showed similar sensitising capacity to intact whey, eliciting capacity was reduced [98]. In the contrary, Roth-Walter et al. concluded that pasteurisation increased sensitising capacity of β-lactoglobulin (BLG) [99].

While as above mentioned immunogenicity, allergenicity and sensitising capacity of EH and HT cow's milk proteins have been already studied in several human and animal studies, to our knowledge other mammalian milks have not been yet subjected for the analysis from the perspective on how processing could influence their immunogenicity, allergenicity, sensitising as well as cross-reactive capacity. Mammalian milks other than cow's milk i.e. goat, sheep, donkey, horse and camel have gained a scientific attention for their usefulness in CMA management [8,100–103]. While goat and sheep due to their high cross-reactivity probably caused by the high homology with cow's milk proteins [21], are not recommended for CMA management [104,105], donkey, horse and camel probably due to the low homology with cow's milk proteins [7], have

been shown to have a higher potential to be used in a management of CMA [8,24,102,106]. For example, in our previous animal study we showed that the immunogenicity and sensitising capacity of cow's and camel milk was comparable however the cross-reactivity was low [21]. Yet, still some rats sensitised to cow's milk showed a cross-reactivity with camel milk. Therefore, in the present study, we evaluated further the impact of processing such as enzyme hydrolysis and heat treatment, on the immunogenicity, sensitising and cross-reactive capacity of cow's and camel milk. This was performed using a high IgE-responder Brown Norway (BN) rat model for intraperitoneal (i.p.) sensitisation with: intact cow's milk, enzyme hydrolysed (EH) cow's milk, heat treated (HT) cow's milk, intact camel milk, EH camel milk and HT camel milk. Antibodies responses were evaluated for quantity, specificity, functionality and cross-reactivity using different enzyme-linked immunosorbent assays (ELISAs), immunoblotting as well as in vivo test.

2. Materials and methods

2.1. Materials

Powder of cow's milk from MlekPol (Grajewo, Poland) was purchased in a local Polish shop. Camel milk powder was kindly provided by Ausnutria Dairy (China) Co., Ltd, (Changsha, Hunan, China). Powders of cow's and camel milk were dissolved in phosphate buffer saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) for further use in this study.

2.1.1. Processed cow's and camel milk products

Preparation of EH cow's and camel milk was as described in details in **Manuscript III**. Briefly, 50 mg/mL of full fat cow's and camel milk proteins were hydrolysed using Pancreatic Trypsin Novo 6.0 S, Type Salt Free (Trp) (Novozymes, Bagsværd, Denmark) and Alcalase 2.4 L FG (Alc) (Novozymes). For preparation of HT cow's and camel milk, 50 mg/mL of full fat cow's and camel milk proteins were heat treated using 121 ^{o}C for 1 h.

2.1.2. Endotoxin content

Endotoxin content was measured by Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (88282, Thermo Fisher, Waltham, MA, US) in accordance with the manufacturers' instruction. While endotoxin level of HT cow's milk, intact camel milk, EH camel milk and HT camel milk was <2 endotoxin units (EU) per mg protein, intact cow's milk and EH cow's milk had an endotoxin level of approx. 9 EU.

2.2. Rats characterisation

In-house breeding colony of BN rats at the National Food Institute, Technical University of Denmark, Denmark were used for animal sensitisation experiment. Rats were kept in macrolon cages at 22 ^{o}C +/- 1^{o}C with 55% +/- 5% relative humidity at a 12 h light-dark cycle and air exchange was applied 8-10 times per hour with overpressure. BN rats were inspected twice a day and

weighted once per week. Rats were kept on a diet free from milk for ≥10 generations. Tap water as well as feed prepared in-house were given *ad libitum*. Animal experiments were conducted at the National Food Institute, Technical University of Denmark. Ethical approval and authorisation was given by the Danish Animal Experiments Inspectorate (2015-15-0201-00553-C1). In addition, experiments was under the supervision of the National Food Institute's in-house Animal Welfare Committee for animal care and use.

2.3. Intraperitoneal sensitisation experiment

BN rats between 4-8 weeks of age were divided into six groups of eight rats (n=four/gender) and housed two per cage. Rats were immunised i.p. in order raise antibodies against cow's milk, EH cow's milk, HT cow's milk, camel milk, EH camel milk, HT camel milk with protein content adjusted to 200 µg in 0.5 mL PBS, without addition of adjuvant at day 0, 14 and 28 (Figure 1). Control group was included in the study were naïve rats received i.p. injections with PBS only. At day 35, oral food challenge (OFC) was performed and rats were sacrificed after 1 h where blood was collected. Blood samples were converted into serum a day after and stored in -20 ˚C until analysis.

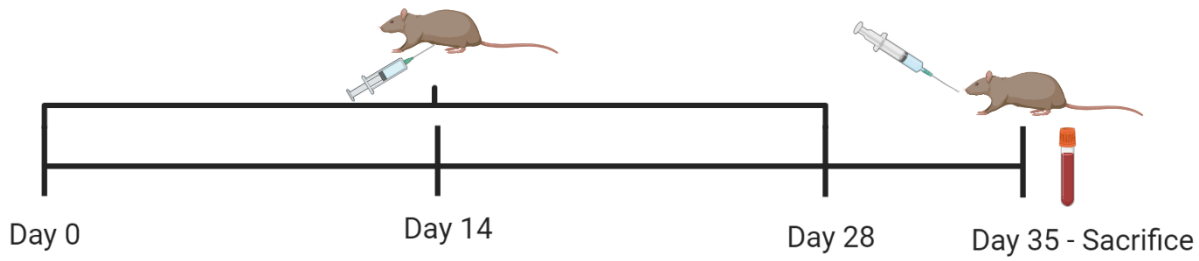

Figure 1. Outline of Intraperitoneal (i.p.) sensitisation experiment. Brown Norway rats were immunised i.p. with intact cow's milk, enzyme hydrolysed (EH) cow's milk, heat treated (HT) cow's milk, intact camel milk, EH camel milk and HT camel milk, three times at day 0, 14 and 28 and with protein content adjusted to 200 µg. At day 35 rats were challenged with 100 mg of intact version of the product they were immunised with, watched for upper gastro-intestinal symptoms, sacrificed after 1 h and blood was collected. Figure created with BioRender.com

2.4. Oral food challenge

To investigate symptoms of an allergic reaction after OFC, rats were challenged intragastrically with 100 mg of proteins in 1 mL PBS using intact version of the product they were immunised with (either intact cow's or camel milk). Rats were video filmed for 10 minutes immediately after OFC and sacrificed 1 h after. Videos with rats' behaviors after OFC were randomised and carefully watched for symptoms episodes count. Types of symptoms observed were upper gastro-intestinal symptoms by means of singultus- and emesis-like behavior, indicating the experience of nausea.

2.5. Indirect ELISA for IgG1

To detect IgG1 antibodies specific for intact cow's milk and camel milk, indirect ELISA was performed as previously described [21]. IgG1 antibodies specific for EH cow's milk, HT cow's milk, EH camel milk and HT cow's milk were detected by using the same previously described protocol, where Maxisorp microtitre plates (96-well, Nunc) were coated with 100 µL/well of 10 µg/mL of EH cow's milk, HT cow's milk, EH camel milk or HT camel milk in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), and incubated overnight at 4 °C. Results are expressed as the log2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control +3 SD.

2.6. Antibody Capture ELISA

To detect IgE antibodies specific for intact cow's milk, EH cow's milk, HT cow's milk, intact camel milk, EH camel milk and HT camel milk antibody capture ELISAs were performed as previously described [21] with few exceptions. Antibody capture ELISA was optimised to use the most proper blocking solution as well as digoxigenin (DIG)-coupled milk product dilution for each of the six milk products. For cow's and camel milk specific IgE detection, 5% (v/v) of horse serum was used for plate blocking step as well as for serum samples dilution. DIG-coupled cow's and camel milk were diluted 1:10. For HT cow's milk, HT camel milk and EH cow's milk specific IgE detection, 10% (v/v) of rabbit serum was used for plate blocking step, serum samples dilution as well as DIG-coupled products 1:100 dilution. For EH camel milk specific IgE detection 5% (v/v) rabbit serum was used for plate blocking step, serum samples dilution as well as DIG-coupled EH camel milk 1:100 dilution. Results are expressed as the log2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control +3 SD.

2.7. Inhibitory ELISA

Inhibitory ELISA was performed in order to evaluate changes in the reactivity pattern towards intact cow's and camel milk proteins caused by processing as previously described [21]. In brief, plates were coated with intact cow's milk, EH cow's milk, HT cow's milk, intact camel milk, EH camel milk and HT camel milk. Subsequently, serum pools of each group, were diluted to achieve an OD of 2.0. Serum pools of each group were pre-incubated with ten-fold serial dilution of the product that each group was immunised with as well as intact version of this product. Serum pools from groups immunised with cow's and camel milk were pre-incubated with ten-fold serial dilution of all products. After 1 h serum-product solutions were added to the plates in duplicates and incubated for 1 h. Inhibitory ELISA was performed three times and results were expressed as percentage of inhibition against the concentration of inhibitor.

2.8. Avidity ELISA

To evaluate binding strength between milk products and specific IgG1, avidity ELISA was performed as previously described [86] with the serum samples of each rat that reached at least OD 0.5 without inhibitor included. Results were expressed as potassium thiocyanate (KSCN) concentration required for 50% inhibition of binding between antibody-antigen.

2.9. Tryptase ELISA

To determine tryptase concentration in serum, rat tpsab1 ELISA kit (EKR864, Nordic BioSite AB, Täby, Sweden) was used in accordance to manufacturers' protocols using pools of serum from each group. The assay was performed two times in duplicates and results were expressed as ng/mL.

2.10. Immunoblotting

To examine IgG1 specificity towards intact cow's and camel milk proteins, immunoblotting was performed using serum pools from rats immunised with intact cow's milk, EH cow's milk, HT cow's milk, intact camel milk, EH camel milk, HT camel milk. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with 5 μg of intact cow's and camel milk proteins was performed as previously described [21], and subsequently proteins were transferred onto polyvinylidene difluoride membranes (Trans-Blot™ Transfer System Mini PVDF Transfer Pack, 1704156, Bio-Rad) and further immunoblotting protocol was followed as previously described [21], with minor changes. Briefly, immunoblots with intact cow's and camel milk proteins were performed using serum pools with adjusted dilution of 1:2,000 (v/v) for rats immunised with cow's milk and 1:1,000 (v/v) for rats immunised with camel milk. Immunoblots only with cow's milk proteins were performed where serum pools from rats immunised with EH cow's milk, 1:100 (v/v) and serum pools from rats immunised with HT cow's milk were used. Further, immunoblots only with camel milk proteins were performed where serum pools from rats immunised with EH camel milk, 1:200 (v/v) and serum pools from rats immunised with HT camel milk, 1:1,000 (v/v) were used. Secondary antibody (horse radish peroxidase (HRP)-labelled-mouse-anti-rat IgG1, 3060-05, Southern Biotech, Birmingham, AL, US) was diluted 1:15,000 and added together with StrepTacin-HRP conjugate (Bio-Rad) diluted 1:20,000 for marker proteins detection to all performed immunoblots. Membranes were further developed as previously described [21].

2.11. Statistics

Graphs and statistical analyses were made using GraphPad Prism version 9.0.1 (San Diego, CA, US). Results from ELISAs were expressed as log2 antibody titre values. All data was first tested for the normal distribution using D'Agostino-Pearson normality test. If the data passed normality test, differences between two groups were analysed using parametric *t-test* while differences between more than two groups were analysed using one-way ANOVA followed by Bonferroni

post-test. If the data did not pass normality test, differences between two groups were analysed using non-parametric Mann Whitney while differences between more than two groups were analysed using Kruskal-Wallis test followed by Dunn's post-test for multiple comparison. Differences were significant if P≤ 0.05. Asterisks indicate statistically significant differences between two groups: *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001 and ns, no statistically significant.

3. Results

To compare inherent immunogenicity, sensitising and cross-reactive capacity of six different milk products, rats were immunised i.p. three times with intact cow's milk, EH cow's milk, HT cow's milk, intact camel milk, EH camel milk, HT camel milk (Figure 1) and subsequently in vivo and ex vivo analyses were performed.

3.1. Impact of processing on immunogenicity

To evaluate inherent immunogenicity of intact cow's and camel milk as well as their EH and HT versions, the level of specific IgG1 in serum samples was evaluated. Control group (PBS immunised rats) did not react with any of the products (data not shown).

The inherent immunogenicity of EH cow's milk and HT cow's milk was significantly lower when comparing with intact cow's milk while the inherent immunogenicity of EH camel milk and HT camel milk did not show any statistically significant difference when comparing with the inherent immunogenicity of intact camel milk (Figure 2A). However, rats immunised with EH camel milk showed slightly lower median value of specific IgG1 when comparing with rats immunised with intact and HT camel milk as displayed in Figure 2A.

Avidity ELISAs were performed to determine binding strength between specific IgG1 and proteins in: intact cow's milk, EH cow's milk, HT cow's milk, intact camel milk, EH camel milk and HT camel milk. Figure 2B displays median amount of potassium thiocyanate (KSCN) that was needed to inhibit 50% of the binding between IgG1 antibody-antigen. Results showed that there was no statistically significant difference in binding strength between IgG1 raised against intact cow's milk and processed versions of cow's milk as well as between intact camel milk and processed versions of camel milk as displayed in Figure 2B. However, camel milk products specific IgG1s were shown to have a higher binding strength then comparing with cow's milk products specific IgG1s, though it was only EH camel milk specific IgG1 that showed a significantly higher avidity when comparing with EH cow's milk specific IgG1 (Figure 2B).

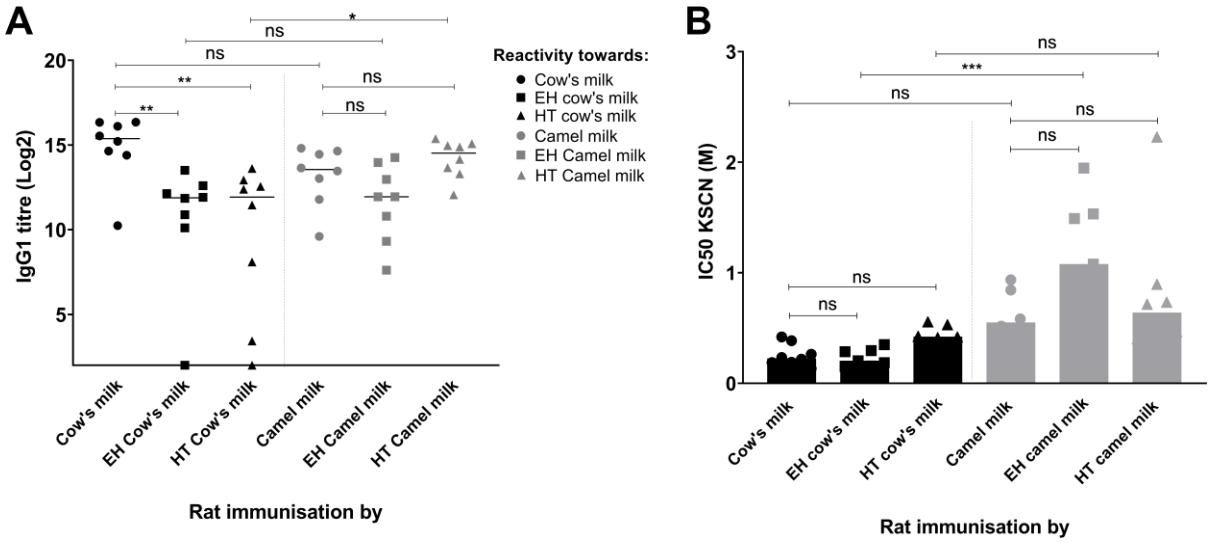

Figure 2. Specific IgG1 antibody responses. (A) Comparison of inherent immunogenicity of (●) intact cow's milk, (■) EH cow's milk, (▲) HT cow's milk, (●) intact camel milk, (■) EH camel milk and (▲) HT camel milk. Each symbol represents single rat and horizontal lines indicate median value. Kruskal-Wallis test followed by Dunn's post-test was applied. Asterisks indicate statistically significant differences when: ∗ = $p \leq 0.05$, ∗∗ = $p \leq 0.01$, ∗∗∗ = $p \leq 0.001$ and ns = no significant. **(B)** Avidity of IgG1 specific for (●) intact cow's milk, (■) EH cow's milk, (▲) HT cow's milk, (●) intact camel milk, (■) EH camel milk and (▲) HT camel milk. Avidity is expressed as potassium thiocyanate (KSCN) concentration for 50% of specific IgG1 inhibition. Kruskal-Wallis test followed by Dunn's post-test was applied and horizontal lines indicate median value. Asterisks indicate statistically significant differences when: ∗ = $p \leq 0.05$, ∗∗ = $p \leq 0.01$, ∗∗∗ = $p \leq 0.001$ and ns = no significant.

3.2. Impact of processing on reactivity pattern and IgG1 specificity

To examine changes in reactivity pattern after processing of intact cow's and camel milk respectively, inhibitory ELISAs were performed. While cow's milk and HT cow's milk were able to fully inhibit IgG1 specific for intact cow's milk, EH cow's milk inhibited approx. 80% of those antibodies (Figure 3A). This may indicate that while heat treatment did not influence immune reactivity of cow's milk proteins, enzyme hydrolysis caused some IgG1-binding epitopes reduction. On the other hand, while intact camel milk was able to fully inhibit IgG1 specific for intact camel milk, neither HT nor EH camel milk fully inhibited those antibodies indicating that immune reactivity of camel milk was influenced by heat treatment and enzyme hydrolysis caused by some IgG1-binding epitopes reduction and masking (Figure 3D). For IgG1 raised against EH cow's milk, while EH cow's milk as expected inhibited fully those antibodies, intact cow's milk reached an approximate inhibition of 80%, indicating formation of new epitopes after enzyme hydrolysis of cow's milk (Figure 3B). That was on the other hand not the case for IgG1 specific for EH camel milk as both intact camel milk and EH camel milk were able to fully inhibit those antibodies indicating that the repertoire of IgG1-binding epitopes was preserved after enzyme hydrolysis of camel milk (Figure 3D). For IgG1 raised against HT cow's milk, both HT cow's milk

and intact cow's milk were able to fully inhibit those antibodies (Figure 3C) and similar results was observed for IgG1 raised against HT camel milk (Figure 3F) indicating that heat treatment of cow's and camel milk did not cause formation of any new IgG1-binding epitopes.

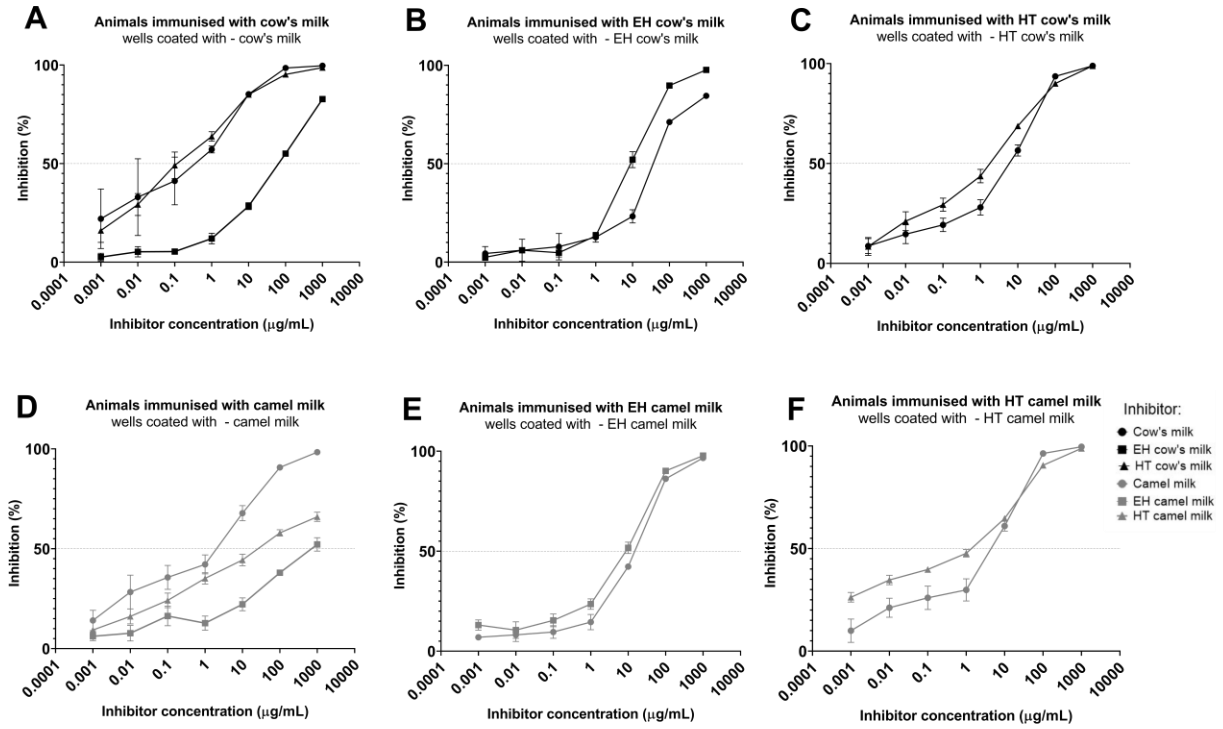

Figure 3. Specific IgG1 binding inhibition. Inhibitory ELISA with (●) intact cow's milk, (■) EH cow's milk, (▲) HT cow's milk, (●) intact camel milk, (■) EH camel milk and (▲) HT camel milk as inhibitors was performed using serum pools from rats immunised with cow's milk, EH cow's milk, HT cow's milk, camel milk EH camel milk and HT camel milk. Each symbol is representation of the percentage of inhibition of IgG1 specific antibodies at different inhibitor concentrations stated on x-axis. Error bars in the inhibition curves represent +/- standard deviation (SD). **(A)** Inhibition curve of sera raised against cow's milk. **(B)** Inhibition curve of sera raised against EH cow's milk. **(C)** Inhibition curve of sera raised against HT cow's milk. **(D)** Inhibition curve of sera raised against camel milk. **(E)** Inhibition curve of sera raised against EH camel milk. **(F)** Inhibition curve of sera raised against HT camel milk.

Further to examine the impact of cow's and camel milk processing on the specificity of the IgG1 responses, immunoblotting was performed. Intact cow's milk specific IgG1 reacted as expected to all major proteins in intact cow's milk such as α-lactalbumin (ALA) around 15 kDa, BLG between 15 and 20 kDa, caseins between 25 and 37 kDa as well as serum albumin (SA) between 50 and 75 kDa and those antibodies also showed cross-reactivity with SA in intact camel milk (Figure 4A). IgG1 raised against EH cow's milk showed reactivity with SA and BLG indicating that those globular proteins were more resistant to hydrolysis than other cow's milk proteins (Figure 4B). IgG1 raised against HT cow's milk showed similar pattern of reactivity towards cow's milk proteins (Figure 4C).

Similarly, intact camel milk specific IgG1 reacted to all major proteins in intact camel milk such as ALA around 15 kDa, caseins between 25 and 37 kDa, and SA between 50 and 75 kDa (Figure 4D). IgG1 raised against EH camel milk only showed reactivity with ALA indicating that this protein in camel milk was more resistant to hydrolysis than other camel milk proteins (Figure 4E). This could also indicate that results on Figure 2B (specific IgG1 antibody avidity) showed that predominant IgG1 specific for EH cow's milk were raised against ALA with a very high avidity. IgG1 raised against HT cow's milk showed similar pattern of reactivity towards cow's milk proteins (Figure 4F).

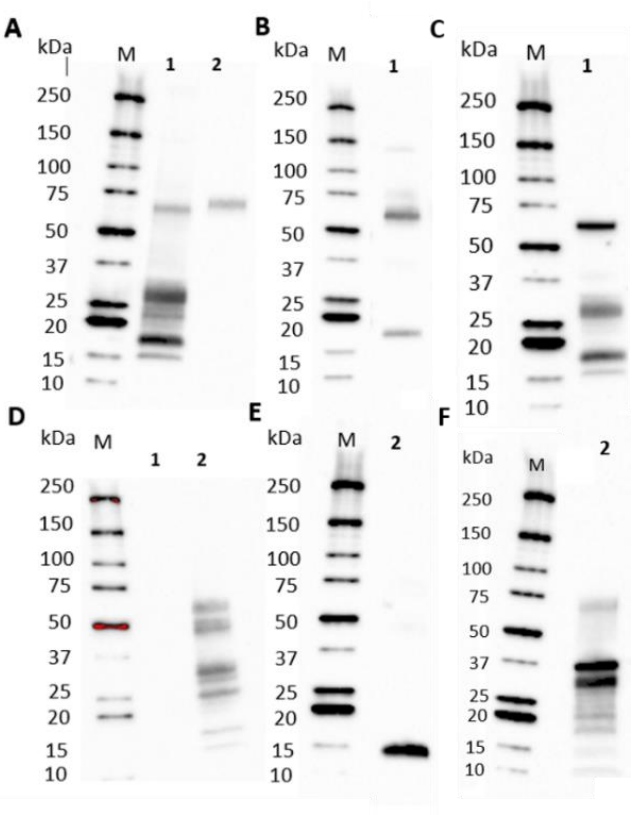

Figure 4. Impact of processing on IgG1 specificity. M, protein standard (kDa); **1,** intact cow's milk; **2,** intact camel milk. **(A)** Comparison of the reactivity of IgG1 specific for intact cow's milk diluted 1:2,000 (*v/v*) towards 5 µg of intact cow's and camel milk proteins. **(B)** Reactivity of IgG1 specific for EH cow's milk diluted 1:200 (*v/v*) towards 5 µg of cow's milk proteins. **(C)** Comparison of the reactivity of IgG1 specific for HT cow's milk diluted 1:1,000 (*v/v*) towards 5 µg of cow's milk proteins. **(D)** Comparison of the reactivity of IgG1 specific for intact camel milk diluted 1:200 (*v/v*) towards 5 µg of cow's and camel milk proteins. **(E)** Comparison of the reactivity of IgG1 specific for EH camel milk diluted 1:200 (*v/v*) towards 5 µg of camel milk proteins. **(F)** Comparison of the reactivity of IgG1 specific for HT camel milk diluted 1:1,000 (*v/v*) towards 5 µg of camel milk proteins.

3.3. Impact of processing on sensitising and eliciting capacity

To evaluate sensitising and eliciting capacity of intact cow's milk, EH cow's milk, HT cow's milk, intact camel milk, EH camel milk and HT camel milk, the level of specific IgE in serum samples was

evaluated. All IgE responses in control group were negative (not shown in the Figure 5A). Due to the differences in sensitivity of antibody-capture ELISA optimised in-house for each product, no statistical analysis could be performed. Both intact cow's and camel milk showed comparable sensitising capacity as except one rat, all rats immunised with intact cow's milk raised IgE specific for intact cow's milk and all rats immunised with intact camel milk raised IgE specific for intact camel milk as shown in Figure 5A. Moreover, while heat treatment of camel milk did not reduce its sensitising capacity as all rats immunised with HT camel milk raised IgE specific for HT camel milk, heat treatment of cow's milk to some extend reduced its sensitising capacity as only half of the rats immunised with HT cow's milk raised IgE specific for HT cow's milk (Figure 5A). Enzymatic hydrolysis on the other hand, caused a complete reduction of sensitising capacity for both cow's and camel milk (Figure 5A). Further, at day 35 OFC was performed to determine eliciting capacity of different milk products, where rats received intact version of the products they were immunised with. Only some rats immunised with intact cow's and camel milk as well as HT camel milk manifested a number of symptoms episodes after OFC (Figure 5B) while rats immunised with EH cow's milk, EH camel milk and HT cow's milk did not show any symptoms manifestation after OFC (Figure 5B). This was aligned with a correlation between specific IgE titre and number of symptom episodes as a strong positive correlation between those two parameters was found (Figure 5C), indicating that the more specific IgE raised, the more symptom episodes after OFC.
Finally, tryptase concentration was measured in pools of serum samples from each group as an allergic reaction marker (mast cells activation marker). Rats immunised with intact cow's milk, HT cow's milk and intact camel milk had a higher concentration of tryptase when comparing with control group (rats immunised with PBS) (Figure 5D). Yet, rats immunised with intact camel milk showed more than 25 times higher concentration of tryptase in their serum samples when comparing with rats immunised with intact cow's milk and HT cow's milk (Figure 9). This might indicate that even though cow's and camel milk showed similar sensitising capacity, their eliciting capacity differed as intact camel milk caused much higher mast cells activation and thus degranulation upon OFC.

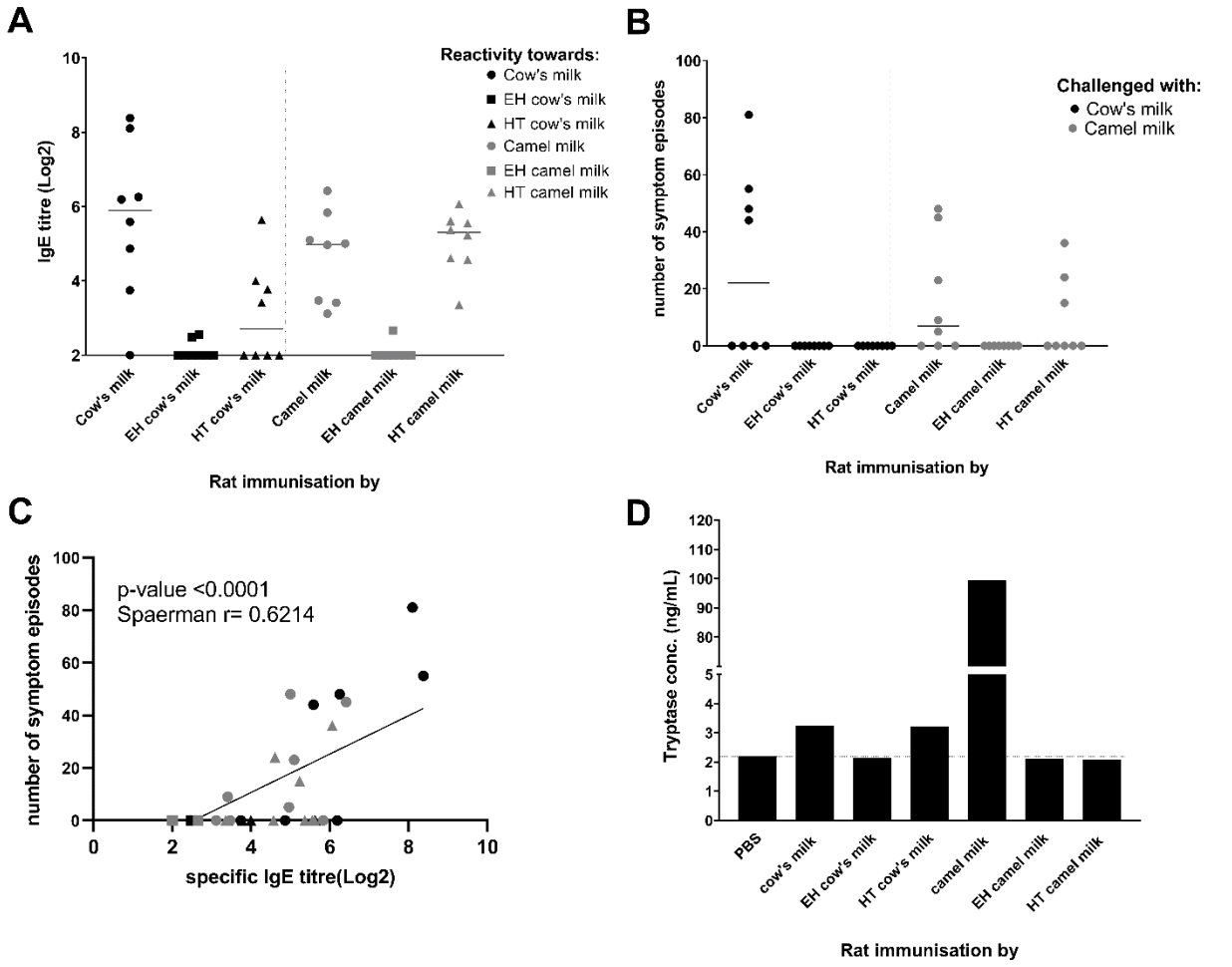

Figure 5. Milk products sensitising and eliciting capacity. (A) Comparison of sensitising capacity of (●) intact cow's milk, (■) EH cow's milk, (▲) HT cow's milk, (●) intact camel milk, (■) EH camel milk and (▲) HT camel milk. Each symbol represents a specific IgE titre value for an individual rat and horizontal lines display median values for each group of rats. **(B)** Number of symptom episodes after oral food challenge (OFC) with (●) intact cow's milk when rats were immunised with cow's milk, EH cow's milk and HT cow's milk and (●) intact camel milk when rats were immunised with camel milk, EH camel milk and HT camel milk. Each symbol represents a number of symptoms episodes for an individual rat and horizontal lines display median values for each group of rats. **(C)** Correlation between specific IgE and number of symptom episodes. Non-parametric Spearman correlations were calculated between all pairs of specific IgE and number of symptom episodes. **(D)** Concentration of tryptase in serum pools from rats immunised i.p. with PBS, cow's milk, EH cow's milk, HT cow's milk, camel milk, EH camel milk and HT camel milk at the day 35 of the experiment. The experiment was performed twice in duplicates and mean value of the concentration is displayed on the graph.

3.4. Impact of processing on cross-reactivity in cow's and camel milk immunised rats

To evaluate whether and how processing such as enzyme hydrolysis and heat treatment influenced reactivity of cow's and camel milk in cow's and camel milk immunised rats, first

inhibitory ELISAs were performed using pools of serum from rats immunised with cow's and camel milk. Both cow's milk and HT cow's milk were able to fully inhibit IgG1 specific for cow's milk while EH cow's milk inhibited approx. 80% of those antibodies, indicating that some IgG1 epitopes were destroyed upon enzyme hydrolysis (Figure 7A). Camel milk on the other hand inhibited only approx. 60% of IgG1 specific for cow's milk and inhibition was even decreased with HT and EH camel milk indicating that cross-reactive epitopes between cow's and camel milk were further destroyed with processing of camel milk (Figure 6A).

Camel milk specific IgG1were fully inhibited only by camel milk as shown in Figure 6B. HT camel milk inhibited only approx. 65% of those antibodies while EH camel milk caused approx. 50% of their inhibition (Figure 6B). This indicates that both heat treatment and enzyme hydrolysis caused a destruction of some IgG1 epitopes. Cow's milk was able to inhibit approx. 60% of the IgG1 specific for camel milk, and inhibition was even decreased with HT and EH cow's milk indicating that cross-reactive epitopes between cow's and camel milk were further destroyed with processing of cow's milk (Figure 6B).

Further to determine changes in reactivity of IgE specific for cow's and camel milk towards cow's and camel milk as well as their processed versions, antibody-capture ELISA was performed using serum from rats immunised cow's and camel milk. Rats sensitised to cow's milk reacted to HT cow's milk but not EH cow's milk, indicating that the allergenicity of cow's milk was reduced after enzyme hydrolysis but heat treatment. IgE specific for cow's milk did not react to any of camel milk products indicating no cross-reactivity (Figure 6C). Rats sensitised to camel milk reacted towards HT camel milk, some to EH camel milk and half of the rats reacted towards HT cow's milk but not cow's milk (Figure 6D). This indicates that probably heat treatment of cow's milk may increase cross-reactivity with camel milk and that intact cow's milk could be a better choice for individuals with camel milk allergy.

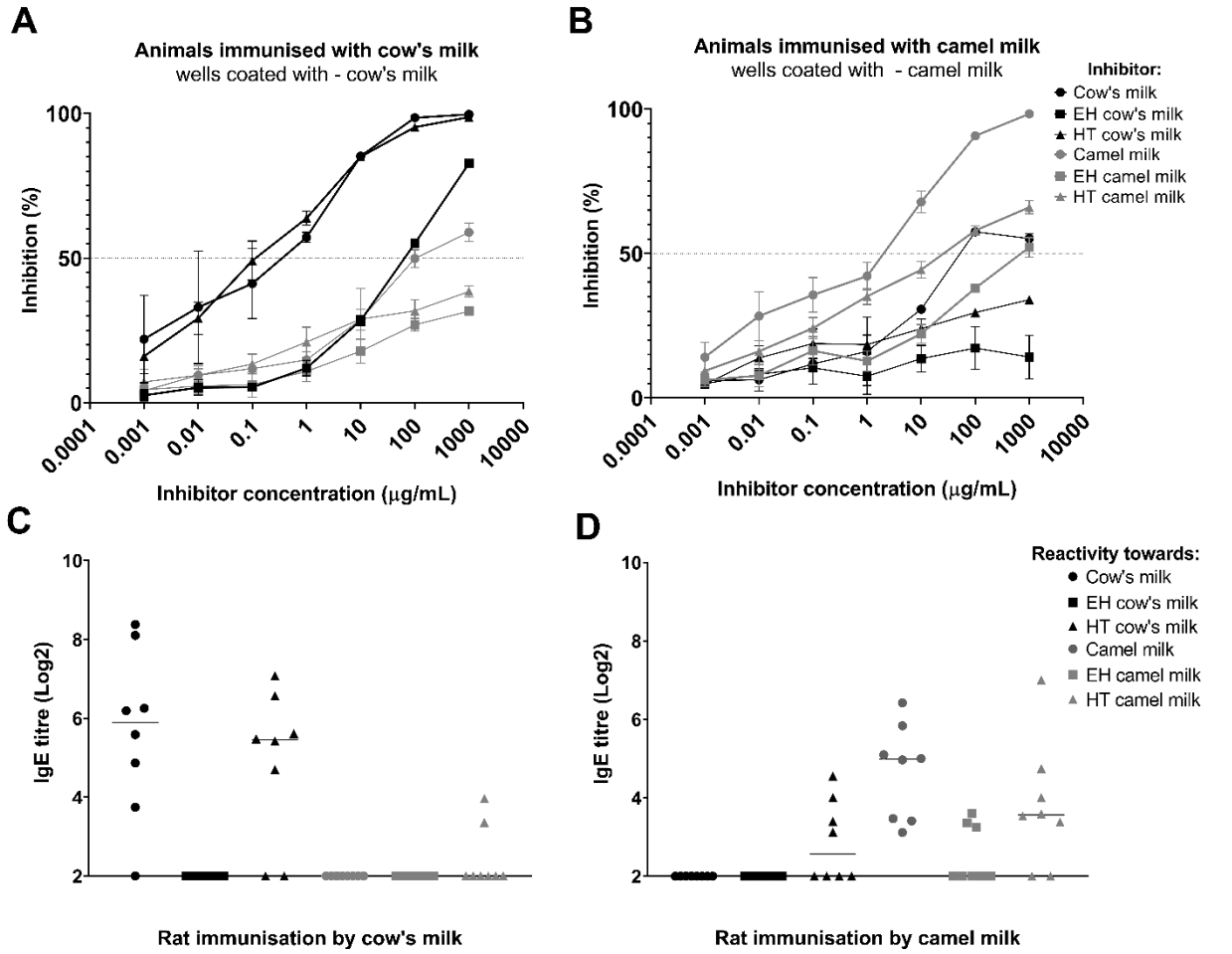

Figure 6. Cross-reactivity of cow's and camel milk proteins in rats immunised with intact cow's and camel milk. Specific IgG1 binding competition using inhibitory ELISA with (●) intact cow's milk, (■) EH cow's milk, (▲) HT cow's milk, (●) intact camel milk, (■) EH camel milk and (▲) HT camel milk as inhibitors was performed using pool of serum from rats immunised with **(A)** cow's milk and **(B)** camel milk. Each symbol represents the percent of inhibition of IgG1 specific antibodies at different concentration of inhibitors. Error bars represent +/- standard deviation (SD) as each measurement was performed in triplicate. IgE specific for (●) intact cow's milk, (■) EH cow's milk, (▲) HT cow's milk, (●) intact camel milk, (■) EH camel milk and (▲) HT camel milk in serum from rats immunised with **(C)** cow's milk and **(D)** camel milk. Each symbol represents a specific IgE titre value for an individual rat and horizontal lines on the graphs display median values for each group of rats.

2. Discussion

Management of CMA in infants and small children is mainly based on the use of hypoallergenic infant formulas when breastfeeding is impossible of insufficient [77]. This are mainly infant formulas based on hydrolysed cow's milk proteins with reduced allergenicity. Yet, there is an increasing interest in alternative protein sources for infant formulas manufacture in general but also dedicated for CMA management and prevention [7]. Alternative protein sources based other mammalian milk proteins need to be evaluated for their immunogenicity, sensitising capacity and

potential cross-reactivity with cow's milk proteins [107]. In our previous study we concluded that cow's and camel milk had comparable immunogenicity and sensitising capacity but their cross-reactivity was low [21]. In the present study we confirmed these results, showing comparable immunogenicity with similar avidity as well as sensitising capacity. In the present study we also confirmed that cross-reactivity between cow's and camel milk proteins was low. Further, we aimed to evaluate how processing such as enzyme hydrolysis and heat treatment influenced cow's and camel milk proteins immunogenicty, sensitising and cross-reactive capacity. In this study we showed that enzyme hydrolysis completely reduced cow's and camel milk proteins sensitising capacity, still maintaining their immunogenicity. These results could be explained by an analysis of EH cow's and camel milk in **Manuscript III**, which showed that no intact proteins were left after enzyme hydrolysis of cow's and camel milk**.** Yet, based on (SDS-PAGE) and gel permeation chromatography (GPC) it was shown that peptides around 5 kDa, thus consisting of approx. 45 aa were maintained.

In this study we showed that heat treatment of cow's milk partially decreased its allergenicity as well as eliciting capacity while no allergenicity was decreased after heat treatment of camel milk. Our results are in line with the study by Graversen et al. which also showed that heat treatment reduced the inherent i.p. sensitising and eliciting capacity of cow's milk whey proteins [98]. This indicated that heat treatment influence allergenicity of cow's and camel milk proteins in a different way.

3. Conclusions

This study showed that heat treatment and enzyme hydrolysis influenced cow's and camel milk immunogenicity, sensitising capacity, reactivity and its specificity in different ways. In addition heat treatment and enzyme hydrolysis showed to further decrease cross-reactivity between cow's and camel milk proteins.

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6 Manuscript V

Camel milk cannot prevent the development of cow's milk allergy – A study in Brown Norway rats

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Camel milk cannot prevent the development of cow's milk allergy – a study in Brown Norway rats

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Abstract

Scope: Currently there are no specific recommendations for the use of any particular infant formula in the prevention of cow's milk allergy (CMA). Recently, there has been an increasing interest in alternative infant formulas based on milk proteins from other sources than the cow, including milk from other mammalians such as goat, sheep, donkey, horse and camel. Whereas these have been studied for their usability in CMA management, there are no studies of their CMA preventive capacity. Thus, the aim of present study was to evaluate whether camel milk could prevent CMA and vice versa.

Methods and results: The capacity of camel milk in preventing CMA and vice versa were evaluated in a well-established prophylactic Brown Norway rat model. IgG1, IgE and IgA responses, allergy elicitation, intestinal gene expression and protein uptake were analysed. The study demonstrated that camel and cow's milk in general had an insignificant cross-preventive capacity. Yet, whereas cow's milk was shown to have a low transient capacity to prevent sensitisation and clinical active camel milk allergy, camel milk did not show this effect for CMA.

Conclusions: This study suggested that due to a lack of cross-tolerance, camel milk cannot be used for CMA prevention.

Keywords

allergy prevention, animal model, camel milk, cow's milk allergy, food allergy, infant formula

1. Introduction

Cow's milk is one of the most common sources of protein causing IgE-mediated food allergy in infants and small children [5]. Cow's milk allergy (CMA) affects 0.5-3.8% of small children, yet the prevalence varies between different countries [5,71]. For CMA management, strict avoidance of intact cow's milk proteins is needed, and for infants not exclusively breastfed the use of hypoallergenic infant formula is recommended [6]. For CMA prevention in infants and small children at high-risk of developing CMA, there are currently no specific recommendations, neither for the use of particular infant formulas if breastfeeding is not possible nor for when to introduce cow's milk proteins, however, delayed introduction of common allergens is discouraged [7,39]. Nevertheless, previously guidelines recommended the use of partially hydrolysed infant formula (pHF) for the prevention of CMA in infants not exclusively breastfed and at high-risk of developing CMA [108,109], but as current evidence from human studies point to no benefit of pHF in preventing CMA, this recommendation has been omitted in the latest guidelines [39,80,81].

A few human studies have assessed the preventive capacity of pHF in infants at high-risk of developing CMA, where Von Berg et al. [110], Vandenplas et al. [111], and Chandra [112] showed the benefit of pHFs in preventing CMA when compared to conventional formulas, while Lowe et al.[83] showed no benefit of pHF in preventing CMA. In line with the study of Lowe et al. [83], three systematic reviews stressed that there is no evidence for a preventive capacity of pHFs [84,113,114]. The capacity of hydrolysed cow's milk products in preventing CMA has also been studied in animal models, and like human studies, with different outcomes. While some studies showed that partially hydrolysed whey proteins as well as hydrolysed casein could prevent sensitisation towards intact whey proteins and intact caseins, respectively, to the same degree as their intact counterpart [115–118], another study showed that partially hydrolysed whey proteins could only partly prevent CMA [119]. Yet, a study investigating the preventive capacity of different whey protein-based hydrolysates with various degree of hydrolysis (DH), showed that the preventive capacity of hydrolysates was dependent on the DH, and that mild hydrolysis conferred the whey-based proteins an event better preventive capacity than the intact counterpart [80]. Thus, based on human as well as animal studies it seems there is no unambiguous evidence for the benefit of using pHF in the prevention of CMA.

There seems to be a growing interest in new types of infant formulas based on non-hydrolysed proteins, both as alternatives to conventional infant formula but also for the prevention and management of CMA [7]. For example, in a study by Graversen et al., investigating the impact of heat-treatment on the preventive capacity of whey protein, it was shown that heat-treated whey protein and the unmodified counterpart were equally good in inducing tolerance towards whey protein but that the heat-treated version had a lower allergenicity [98].

Alternative infant formulas based on other protein sources than cow's milk, like milk from other mammalians, have gained an increasing interest in the recent decade [7]. Especially milk

from goat, sheep, donkey, horse and camel has gained an interest as alternative protein sources in infant formulas [24,100,106,120–122]. Over the last years, there has been an increasing research interest in camel milk for its use in infant formulas, and especially for its utility in CMA management [21,26,103,123]. This is mainly attributed to the low homology between camel and cow's milk proteins [19,24,103], as well as due to the lack of β-lactoglobulin (BLG) in camel milk, which is one of the major allergens in cow's milk [23]. In a recent animal study, we showed that cow's and camel milk possessed similar inherent immunogenicity and allergenicity, but that the cross-reactivity between counterpart proteins was low [21]. In line, several studies analysing blood samples from cow's milk allergic children showed no or low IgE binding to camel milk proteins confirming low cross-reactivity between cow's and camel milk proteins [8,25,124,125]. Further, in human trials it has been shown that camel milk is well tolerated by the majority of cow's milk allergic children, stressing its potential in CMA management [24,26,103]. However, whether or not early introduction of camel milk could drive a potential future tolerance to cow's milk proteins has to our knowledge not been investigated. Therefore, in the present study, we assessed the capacity of cow's and camel milk in inducing cross-tolerance and investigated whether camel milk could prevent CMA and whether cow's milk could prevent camel milk allergy. For this purpose, a well-established prophylactic Brown Norway (BN) rat model was used [98,116,118].

2. Experimental Section

2.1. Milk Products

Cow's milk powder (MlekPol, Grajewo, Poland) was purchased in a local Polish shop, while camel milk powder was kindly provided by Ausnutria Dairy (China) Co., Ltd, (Changsha, Hunan, China). Solutions of the products were prepared by dissolving cow's and camel milk powders in sterile PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) to a protein concentration of 50 mg/mL and stored at -20 ^{o}C until use. Endotoxin content was measured by Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (88282, Thermo Fisher, Waltham, MA, US) in accordance with the manufacturer's instruction. The endotoxin level of camel milk was <2 endotoxin units (EU) per mg protein, while the endotoxin level of cow's milk was ~9 EU per mg of protein.

2.2. SDS-PAGE

In order to evaluate cow's and camel milk protein profiles, SDS-PAGE was performed as previously described [21], with minor changes. Briefly, cow's and camel milk proteins (15 μg) were separated under reducing conditions using a 4-20% precast polyacrylamide gel (Mini-Protean TGX Stain-Free gel, 4568094, Bio-Rad). Proteins were visualised by Bio Safe™ Coomassie (1610786, Bio-Rad) and photographed using the Imager ChemiDoc XRS+ (Bio-Rad).

2.3. Rats

BN rats from the in-house breeding colony at the National Food Institute, Technical University of Denmark were kept in macrolon cages (n=2/cage) at 22 ^{o}C +/- 1 ^{o}C with 55 +/- 5% relative humidity and with a 12 h light-dark cycle. Rats were inspected once a day and weighted once a week. Rats were kept on an in-house prepared diet free from milk proteins, with rice, fish and potato as protein sources, for >10 generations. Diet and water were given *ad libitum*.
The animal experiment was carried out at the National Food Institute, Technical University of Denmark under ethical approval given by the Danish Animal Experiments Inspectorate and the authorisation number 2020-15-0201-00500-C1. The experiment was overseen by the Technical University of Denmark's in-house Animal Welfare Committee for animal care and use.

2.4. Dosage regimen

To evaluate the capacity of cow's and camel milk in preventing CMA and camel milk allergy, an animal experiment was performed. The animal experiment was divided into two phases; an intervention phase and a post-immunisation phase which was completed with in vivo tests, as displayed in Figure 1. A total of 48 BN rats, 4-7 weeks of age, were divided into six groups of 8 rats (n=4/gender). For investigating prevention of CMA, groups of rats (Group 1-3) received either water as a control, cow's milk or camel milk *ad libitum* in their drinking bottles for 21 days (Intervention phase: Day 0-20) for the purpose of inducting oral tolerance (Figure 1). Similarly, for investigating prevention of camel milk allergy, groups of rats (Group 4-6) received either water as a control, cow's milk or camel milk *ad libitum* in their drinking bottles for 21 days (Intervention phase: Day 0-20) for the purpose of oral tolerance induction (Figure 1). The milk protein concentration in the drinking bottles was 12.5 g protein/L. Subsequently, after one week of rest, rats were post-immunised intraperitoneally (i.p.) with either 100 µg of cow's milk proteins (Group 1-3) or 100 µg of camel milk proteins (Group 4-6) in 0.5 mL PBS, once a week for four weeks (Post-immunisation phase: Day 28, 35, 42 and 49). The animal experiment was completed with two in vivo tests, where an ear swelling test was performed at Day 53, and an oral food challenge (OFC) was performed at Day 56 an. Rats were sacrificed one week after last post-immunisation at Day 56 by exsanguination using carbon dioxide inhalation as anaesthesia and blood was collected. Throughout the experiment, blood samples were collected from the sublingual vein; after the intervention phase at Day 28 and one week after each post-immunisation at Day 35, 42, 49 and 53. In addition, faecal samples were collected at Day 0, 28 and 56. At sacrifice (Day 56) the following samples were collected; small intestine (SI) content, mesenteric lymph nodes (mLN), pieces of SI, lamina propria (LP), epithelium (EPI) and Peyer's patches (PP). Blood samples were converted into serum, and faecal and SI content samples were converted into fecal and SI content water, respectively, as previously described [126].

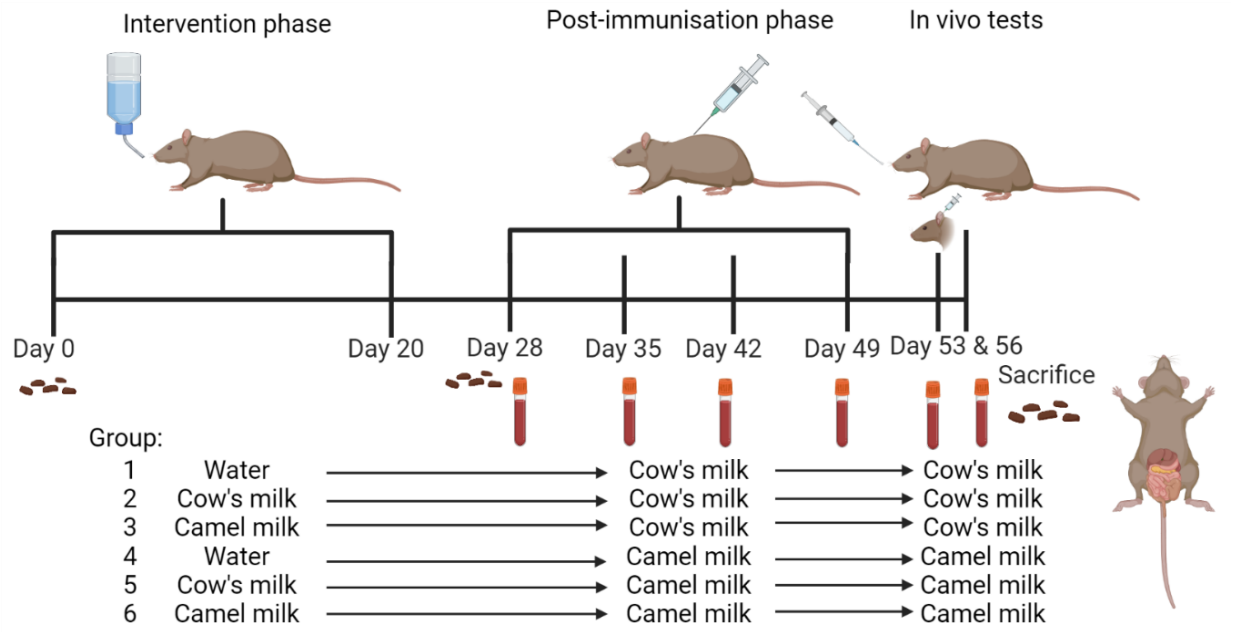

Figure 1. Outline of primary prevention animal experimental design. In the intervention phase (Day 0-20), Brown Norway (BN) rats were *ad libitum* administered with water as a control, cow's milk or camel milk in their drinking bottles for 21 days. Subsequently, in the post-immunisation phase, the BN rats were intraperitoneally (i.p.) immunised with either cow's milk or camel milk for a total of four times at a one-week interval (Day 28, 35, 42 and 49). At Day 53 an ear swelling test and at Day 56 oral food challenge (OFC) were performed with either cow's milk or camel milk, corresponding to the post-immunisation and subsequently sacrificed. Blood samples were collected at Day 28, 35, 42, 49, 53 and 56, whereas faecal samples were collected at Day 0, 28 and 56. Samples of small intestine (SI) content, mesenteric lymph nodes (mLN), pieces of SI, lamina propria (LP), epithelium (EPI) and Peyer's patches (PP) were collected at the day of sacrifice (Day 56). Figure created with BioRender.com.

2.5. In vivo tests

At Day 53 an ear swelling test was performed as previously described [127]. Briefly, rats were anaesthetised with hypnorm-midazolam and the initial ear thickness was measured. Subsequently, rats were intradermally (i.d.) injected with 20 µL of PBS with either 10 µg of cow's milk protein (Group 1-3) or 10 µg of camel milk protein (Group 4-6) into one ear. Ear thickness was measured again 15 min after injection, and delta ear swelling was calculated for each animal. Further, at Day 56, an OFC was performed, where rats from group 1-3 were intragastrically (i.g.) challenged with 1 mL of PBS with 100 mg of cow's milk protein and rats from group 4-6 were i.g. challenged with 1 mL of PBS with 100 mg of camel milk protein. Rats were observed for 10 min in order to monitor number of upper gastro-intestinal symptoms by means of singultus- and emesis-like behavior, indicating the experience of nausea.

2.6. Indirect ELISA for specific IgG1 detection

To detect serum IgG1 specific for either cow's or camel milk, indirect ELISAs were performed, as previously described [21]. Results are expressed as the log2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control +3 SD.

2.7. Indirect ELISA for specific IgA detection

To detect serum IgA specific for either cow's milk or camel milk, indirect ELISAs were performed. Maxisorp microtitre plates (96-well, Nunc, Roskilde, Denmark) were coated with 100 µL/well of 10 µg/mL of cow's milk or camel milk in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), and incubated overnight at 4 °C. Plates were washed five times between each step in PBS with 0.01 % (w/v) Tween 20 (P1379, Sigma-Aldrich) (PBS-T). For all steps that required incubation, plates were incubated for 1 h in the dark at RT with gentle agitation. First, plates were incubated with 50 µL/well of two-fold serial dilutions of serum samples (v/v) in PBS-T. In each plate, positive and negative control sera were included in order to identify potential plate-to-plate variance. For IgA detection, 50 µL/well of HRP-labelled goat-anti-rat IgA (STAR 111P, Bio-Rad) diluted 1:5,000 (v/v) in PBS-T was added to the plates. Next, plates were additionally washed twice with tap water. To visualise the detection of specific IgA, 100 µL/well of TMB-one (3,3',5,5'-tetramethylobenzidine, 4380A, Kementec Diagnosis, Taastrup, Denmark) was added and incubated for 12 min at RT. The reaction was stopped by adding 100 µL/well of 0.2 M H_2SO_4, and the absorbance was measured at 450 nm with a reference wavelength of 630 nm using a microtitre reader (Gen5, BioTek, EL800 Instrument, Winooski, VT, US). Results are expressed as the log2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control +3 SD.

2.8. Sandwich ELISA for total IgA detection

To detect total IgA in serum, faecal water, and SI content water, sandwich ELISA was performed as previously described [28]. Results are expressed as the log2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control +3 SD.

2.9. Antibody-capture ELISA for specific IgE detection

To detect serum IgE specific for cow's or camel milk, antibody-capture ELISAs were performed as previously described [21], except of 5% (v/v) horse serum (S0910-500, Biowest, Nuaille, France) used as blocking agent. Further, for both products, serum samples were two-fold diluted in 5% (v/v) horse serum. DIG-coupled cow's or camel milk protein were used with a final concentration of 0.03 µg/mL in PBS-T for specific IgE detection. Results are expressed as the log2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control +3 SD.

2.10. Immunoblotting

To detect immune reactive proteins, immunoblotting with serum pools from each group of rats was performed. In brief, SDS-PAGE with 5 µg of cow's and camel milk proteins were performed, and proteins transferred onto polyvinylidene difluoride (PVDF) membranes as previously described [21]. Serum pools from the day of sacrifice (Day 56) were diluted 1:3,000 (v/v), whereas the secondary antibody was diluted 1:15,000 and added together with StrepTacin-HRP conjugate (Bio-Rad) diluted 1:20,000 for protein standard visualisation. All membranes were developed for 15 s for optimal visualisation and direct comparison.

2.11. Tissue RNA extraction, cDNA synthesis, and RT-qPCR

To evaluate expression of different genes of interest, mLN and 2x 0.5 cm pieces of SI, harvested 27 cm and 37 cm distal from the stomach respectively were collected and stored in RNAlater (Invitrogen, Carlsbad, CA, US) at -20 °C. RNA extraction, cDNA synthesis and RT-qPCR were performed as previously described [126]. For RNA from mLN extraction, QIAzol Lysis Reagent (79306, Qiagen, Hilden, Germany) and RNeasy Lipid Tissue Mini Kit (74804, Qiagen) were used in accordance with manufacturers' protocol. Taqman gene assays (Applied Biosystems, Thermo Fisher Scientific, MA, US) used were: Ocln (occludin Rn00580064_m1), TSLP (thymic stromal lymphopoietin, Rn01761072_m1), IL-1β (interleukin 1beta, Rn00580432_m1), IL-4 (interleukin 4, Rn01456866_m1), IL-10 (interleukin 10, Rn01483989_m1), INF-γ (interferon gamma, Rn00594078_m1), TGF-β (transforming growth factor beta, Rn005720_m1), FoxP3 (forkhead box P3, Rn01525092_m1), and CX3XR1 (C-X3-C chemokine receptor 1, Rn00591798_m1). The levels of different gene expression are shown as the relative gene expression by means of $2^{-\Delta CT}$ method using B2m (Beta-2-microglobulin Rn00560865_m1) and Sdha (Succinate dehydrogenase complex Rn00590475_m1) as normalisation genes.

2.12. In vivo intestinal protein uptake

To evaluate protein uptake in the SI compartments, LP, SI content, PP and EPI of BN rats challenged with cow's milk (Group 1-3) were harvested as previously described [98]. BLG ELISA kit (Bethyl Laboratories, Montgomery, TX, US) was used for determination of concentration of BLG, as a marker for cow's milk protein uptake, in supernatants prepared from tissue homogenates in accordance to manufacturers' protocol, as previously described [98].

2.13. Statistical analysis

Graphs and statistical analyses were made using GraphPad Prism version 9.0.1 (San Diego, CA, US). Results from ELISAs are expressed as log2 antibody titre values. All data were initially tested for normal distribution using D'Agostino-Pearson normality test. If the data passed normality test, differences between two groups were analysed using parametric *t-test* while differences between more than two groups were analysed using one-way ANOVA followed by Bonferroni

post-test. If the data did not pass normality test, differences between two groups were analysed using non-parametric Mann Whitney test while differences between three or more groups were analysed using Kruskal-Wallis test followed by Dunn's post-test for multiple comparison. Differences were significant if P≤ 0.05. Asterisks indicate statistically significant differences between two groups: *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001 and ns indicates no statistically significant difference.

3. Results

3.1. Humoral immune responses

To evaluate the humoral immune responses developed against cow's or camel milk proteins throughout the study, levels of specific IgG1 were measured at six different time points. At first, it was shown that *ad libitum* bottle administration of both cow's and camel milk promoted the development of specific IgG1 during the intervention phase, as rats administered with cow's milk developed a statistically significant cow's milk-specific IgG1 response and rats administered with camel milk developed a statistically significant camel milk-specific IgG1 response, when comparing with water administered rats (Figure 2A,B). A statistically significant lower and non-significant cross-reactive immune response was observed after the intervention phase for rats intervened with camel milk and tested against cow's milk when compared to cow's milk and water intervened rats, respectively, where it was shown that most camel milk intervened rats had IgG1 that could also react with cow's milk (Figure 2A). Similarly, a statistically significant lower and non-significant cross-reactive immune response was observed for rats intervened with cow's milk and tested against camel milk when compared to camel milk and water intervened rats, respectively, where fewer cow's milk intervened rats had IgG1 that could also react with camel milk (Figure 2B).

Subsequently, the progression of the immune responses upon each of the post-immunisations with either cow's milk or camel milk was assessed, demonstrating that levels of IgG1 specific for cow's milk (Figure 2A,C) or camel milk (Figure 2B,D) increased until reaching a plateau after the second or third post-immunisation. Yet, for rats post-immunised with cow's milk, the cow's milk-specific IgG1 response was statistically significant lower in rats intervened with cow's milk compared to rats intervened with either water or camel milk at sacrifice (Figure 2A). In contrast, such differences were not seen between groups of rats post-immunised with camel milk (Figure 2B).

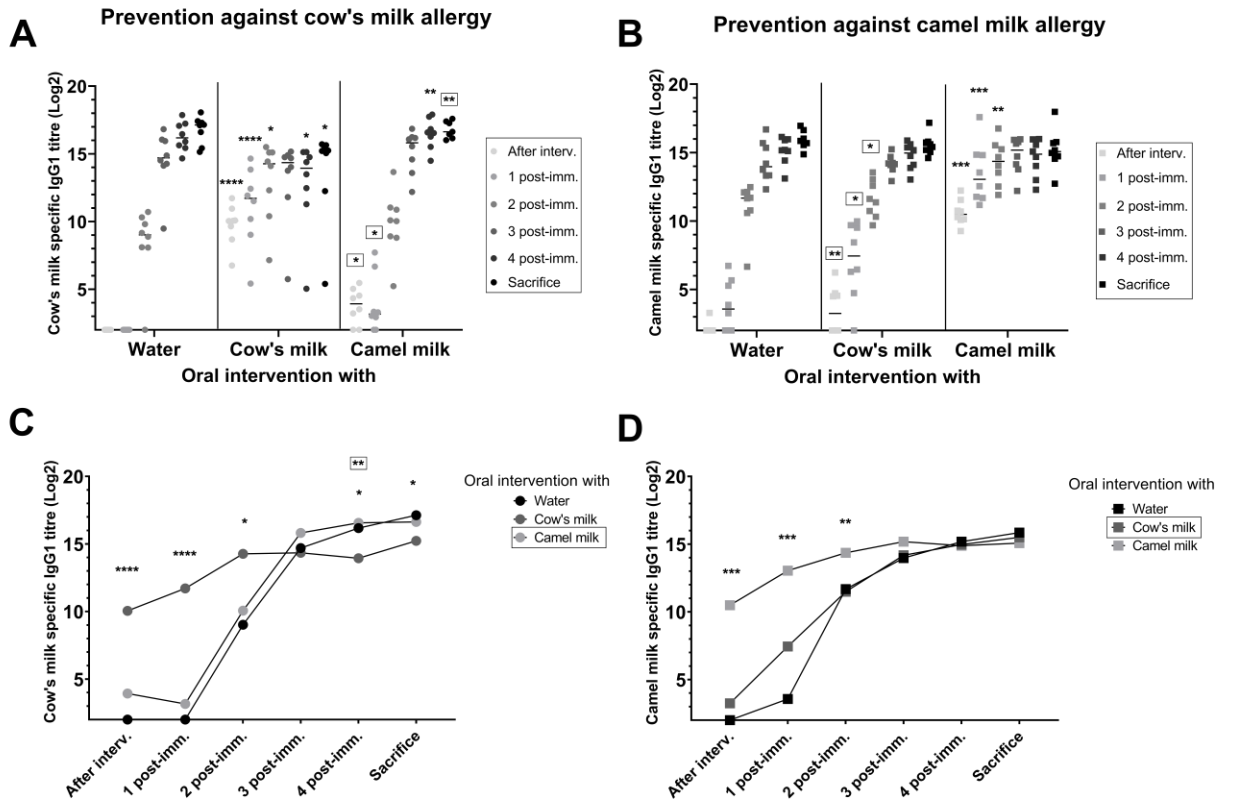

Figure 2. Specific IgG1 responses. (A) IgG1 specific for cow's milk in groups intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(B)** IgG1 specific for camel milk in groups intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. **(A,B)** Each symbol represents individual rats at different time points of the experiment, and horizontal lines indicate median values. **(C)** Progression of the cow's milk-specific immune responses after oral interventions with either: ● water, ● cow's milk, or ● camel milk and subsequent post-immunisations with cow's milk. **(D)** Progression of the camel milk-specific immune responses after oral interventions with either: ■ water, ■ cow's milk, or ■ camel milk and subsequent post-immunisations with camel milk. **(C,D)**. Each symbol represents median value of different groups at different time points of the experiment. Kruskal-Wallis test followed by Dunn's post-test was applied, where statistically significant differences to the water intervened group (unframed) and relative to **(A)** cow's milk or **(B)** camel milk intervened group (framed), or between **(C)** cow's milk or **(D)** camel milk and water intervened group (unframed), and between **(C)** camel milk and **(D)** cow's milk and water intervened group (framed) are shown as *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001. Interv., intervention; post-imm., post-immunisation.

3.2. Prevention of sensitisation

To evaluate the capacity of cow's and camel milk in preventing sensitisation to both cow's and camel milk, levels of specific IgE were measured throughout the animal experiment. Neither *ad libitum* bottle administration with cow's nor camel milk induced sensitisation in the intervention phase, as specific IgE could not be detected in any of the groups (Figure 3A,B). During the post-immunisation regime it was revealed that cow's milk was the most efficient in preventing cow's

milk sensitisation (Figure 3A,C), whereas camel milk was the most efficient in preventing camel milk sensitisation (Figure 3B,D). In fact, whereas cow's milk was efficient in preventing sensitisation towards cow's milk to a statistically significant degree after the third and fourth post-immunisation, camel milk did not show any capacity to prevent sensitisation to cow's milk, as at no time point was camel milk capable of inhibiting the development of cow's milk specific IgE when compared to intervention with water (Figure 3A,C). Similarly, camel milk was efficient in preventing camel milk sensitisation to a statistically significant degree after the third and fourth post-immunisation, whereas cow's milk seemed only to have a low capacity to prevent sensitisation toward camel milk (Figure 3B,D). Yet, in the cow's milk intervened rats a transient reduction in the camel milk-specific IgE level compared to the water intervened rats was observed after the fourth post-immunisations (p=0.0482, Mann-Whitney test between cow's milk and water intervened groups).

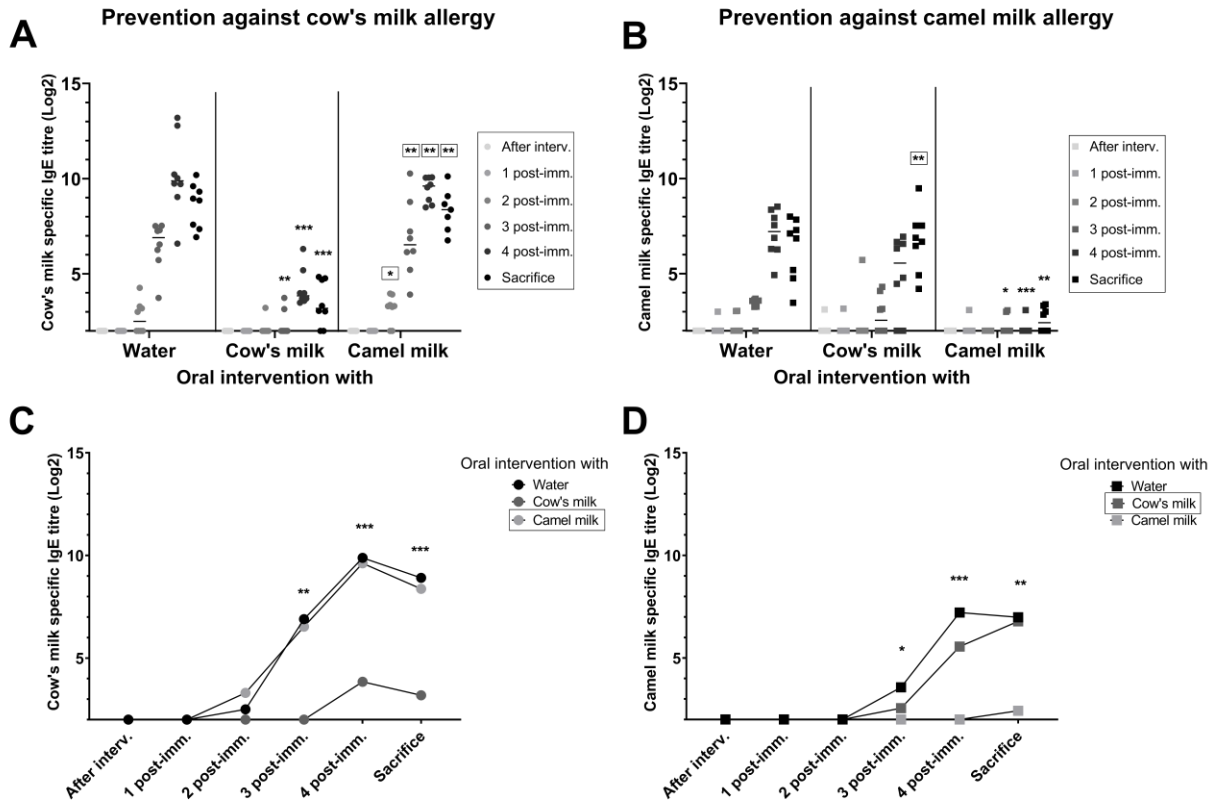

Figure 3. Specific IgE responses. (A) IgE specific for cow's milk in groups intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(B)** IgE specific for camel milk in groups intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. **(A,B)** Each symbol represents individual rats at different time points of the experiment, and horizontal lines indicate median values. **(C)** Progression of the cow's milk-specific IgE responses after oral interventions with: ● water, ● cow's milk, or ● camel milk and subsequent post-immunisations with cow's milk. **(D)** Progression of the camel milk-specific IgE responses after oral interventions with either: ■ water, ■ cow's milk, or ■ camel milk and subsequent post-immunisations with camel milk. **(C,D)** Each

symbol represents median value of different groups at different time points of the experiment. Kruskal-Wallis test followed by Dunn's post-test was applied, where statistically significant differences to the water intervened group (unframed) and relative to **(A)** cow's milk or **(B)** camel milk intervened group (framed), or between **(C)** cow's milk or **(D)** camel milk and water intervened group (unframed), and between camel milk **(C)** and cow's milk **(D)** and water intervened group (framed) are shown as *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001. Interv., intervention; post-imm., post-immunisation.

3.3. Prevention of clinical responses

To evaluate the capacity of cow's and camel milk in preventing clinical responses towards cow's and camel milk, an ear swelling test and an OFC were performed. In the ear swelling test, groups post-immunised with cow's milk, and hence tested for prevention of CMA, showed no statistically significant differences in ear swelling after i.d. injection of cow's milk regardless of the oral intervention (Figure 4A). However, the group intervened with cow's milk showed a slightly lower but non-significant ear swelling when compared with groups intervened with either water or camel milk. Contrary, groups post-immunised with camel milk, and hence tested for prevention of camel milk allergy, showed statistically significant differences in their ear swelling responses. A significantly lower ear swelling after i.d. injection of camel milk was observed for groups intervened with camel milk and cow's milk when compared to the group intervened with water (Figure 4B). These results were aligned with the specific IgE results, and a statistically significant positive correlation between specific IgE levels and delta ear thickness was observed, stressing the functional relevance of the specific IgE raised in the rats (Figure 4C).

The number of symptom episodes was counted after OFC with the product the rats were post-immunised with. The number of symptom episodes after OFC with cow's milk was statistically significant lower for the group intervened with cow's milk in comparison to the groups intervened with either water or camel milk (Figure 4D). Similarly, the number of symptom episodes after OFC with camel milk was statistically significant lower for the group intervened with camel milk in comparison to the groups intervened with either water or cow's milk (Figure 4E). These results were also clearly aligned with the specific IgE results, where a statistically significant positive correlation was observed between specific IgE and number of symptom episodes (Figure 4F), confirming that levels of specific IgE were indicative for the clinical relevance of the allergy. Thus, the results demonstrated that neither could camel milk prevent sensitisation nor clinical reactions against cow's milk, whereas cow's milk had a low capacity to prevent sensitisation and clinical reactions against camel milk.

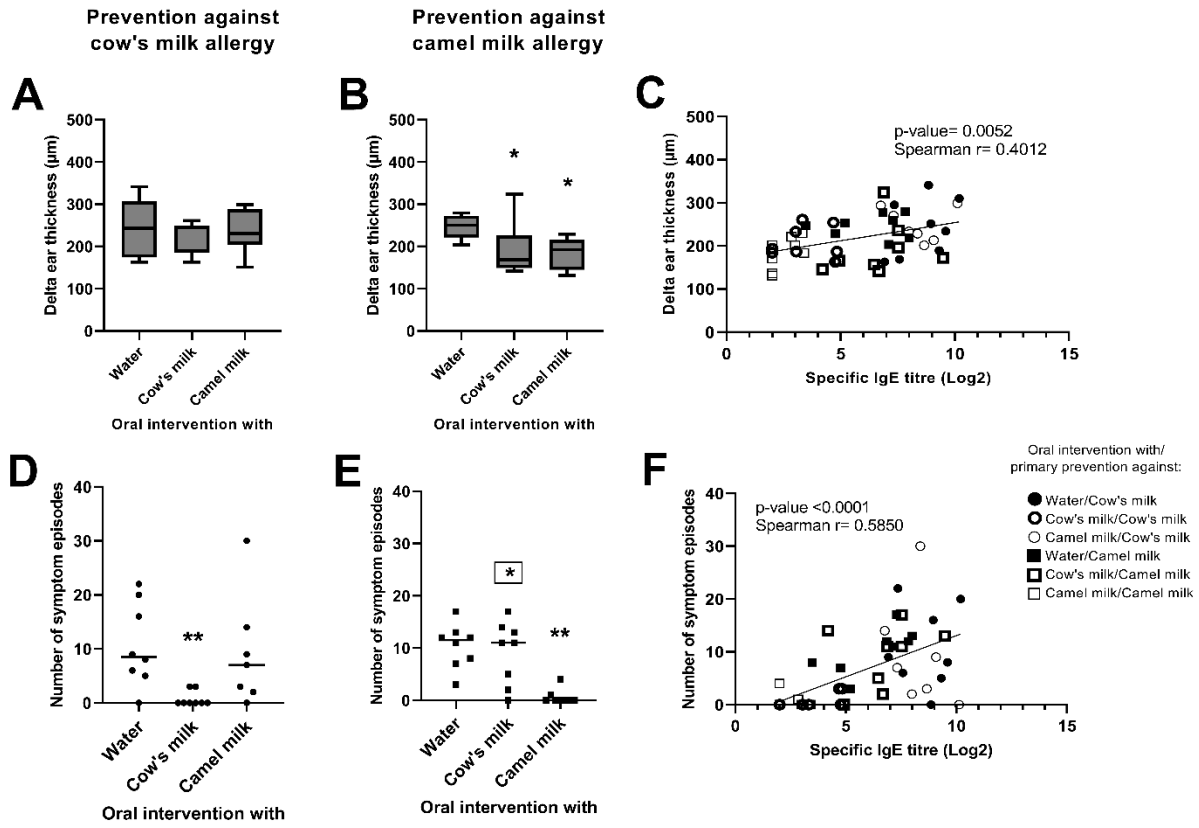

Figure 4. Clinical responses. (A) Ear swelling test with cow's milk in groups intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(B)** Ear swelling test with camel milk in groups intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. **(C)** Correlation between IgE specific for the product rats were post-immunised with and delta ear thickness after intradermal injection with the same product. **(D)** Symptom episodes after oral food challenge (OFC) with cow's milk in groups intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(E)** Symptom episodes after OFC with camel milk in groups intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. **(F)** Correlation between IgE specific for the product rats were post-immunised with and number of symptom episodes after OFC with the same product. **(C-F)** Each symbol represents a single rat, and **(A,B,D,E)** horizontal lines indicate median value. **(A,B,D,E)** Kruskal-Wallis test followed by Dunn's post-test was applied. Statistically significant differences to the water intervened group (unframed) (**A,B,D,E**) and relative to cow's milk intervened group (**A,C**) or camel milk intervened group (**B,D**) (framed) are shown as *P≤ 0.05, **P≤ 0.01. Non-parametric Spearman correlations were calculated between all pairs of specific IgE titre and delta ear thickness or number of symptom episodes (**C,F**).

3.4. IgG1 milk protein specificity

In order to evaluate the specificity of IgG1 and the cow's and camel milk protein binding profiles, immunoblotting was performed.

SDS-PAGE was first performed for protein visualisation using 15 µg of cow's and camel milk protein (Figure 5A). For both cow's and camel milk, caseins were shown as thick bands between

25 and 37 kDa. In addition, α-lactalbumin (ALA) was shown for both products as a band corresponding to the molecular weight of ~15 kDa. A thick band between 15 and 20 kDa was only visible for cow's milk, representing BLG, which is not present in camel milk. Finally, both cow's and camel milk had a band visible just below 75 kDa, corresponding to serum albumin (SA).

Further, SDS-PAGEs with 5 µg of cow's and camel milk protein were performed, transferred to PVDF membranes for immunoblotting where serum pool and secondary antibody dilutions were kept constant for direct comparison.

For rats post-immunised with cow's milk and hence tested for prevention against CMA, it was clearly shown that the reactivity of IgG1 was lower for the group intervened with cow's milk compared to that of the water and camel milk intervened rats (Figure 5B). Bands around 15 kDa representing reactivity with ALA and between 15 and 20 kDa representing reactivity with BLG, were the only bands detected and with a clearly lower intensity for rats intervened with cow's milk compared to rats intervened with either water or camel milk that in contrast showed reactivity towards all major cow's milk proteins (Figure 5B), indicating that the humoral immune response towards SA and caseins were more readily restrained compared to ALA and BLG.

Similarly, for rats post-immunised with camel milk and hence tested for prevention against camel milk, it was shown that the reactivity of IgG1 milk was slightly lower for the group intervened with camel compared to water and cow's milk intervened rats (Figure 5C). Bands around 15 kDa representing reactivity with ALA and between 25 and 37 kDa representing reactivity with caseins, were the bands detected and with a lower intensity for rats intervened with camel milk compared to rats intervened with water or cow's milk that in contrast showed reactivity towards all major camel milk proteins (Figure 5C). Interestingly, a weak band around 15 kDa was visible in cow's milk intervened rats but not in water or camel milk intervened rats, indicating that a solid IgG1 response specific for cow's milk ALA was raised already in the intervention phase (Figure 5C).

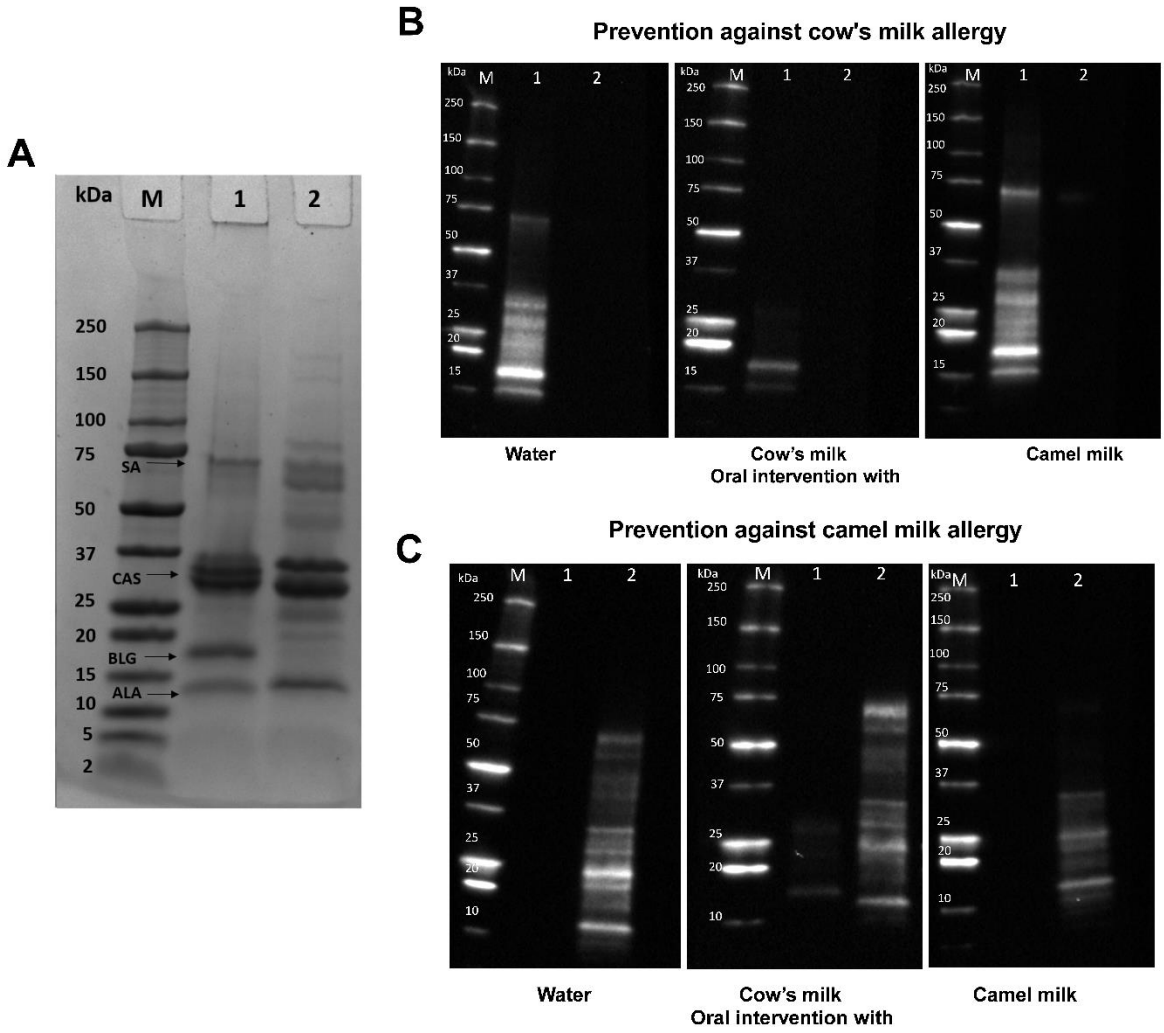

Figure 5. Specificity of IgG1 responses. (A) SDS-PAGE for visualisation of cow's (lane 1) and camel milk (lane 2) protein profiles. M, molecular weight standard marker; kDa, kilodalton; ALA, α-lactalbumin; BLG, β-lactoglobulin; CAS, caseins; SA, serum albumin. **(B)** Immunoblotting to visualise specificity of IgG1 responses towards cow's milk (lane 1) and camel milk (lane 2) in rats intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(C)** Immunoblotting to visualise specificity of IgG1 responses towards cow's milk (lane 1) and camel milk (lane 2) in rats intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy.

3.5. Intestinal immune responses

The level of total IgA was determined in serum, faeces and SI content. In general, for all groups of rats, the total IgA in serum increased throughout the study as the total IgA level was shown to be statistically significant higher at sacrifice compared to after the intervention phase (Figure S1A,B, Supporting Information), indicating a general impact of either age or the post-immunisations on the total IgA level in serum. There were no statistically significant differences between total IgA levels in serum after the intervention phase between the water and cow's or camel milk intervened rats (Figure 6A,C). Contrary, a higher ($p = 0.0004$ and $p=0.1466$, based on

one-way ANOVA) total IgA level was observed for rats intervened with camel milk compared to rats intervened with water and post-immunised with cow's and camel milk, respectively, indicating an effect of the camel milk intervention revealed upon post-immunisations (Figure 6B,D). Further, rats intervened with camel milk had a statistically significant higher level of IgA than cow's milk intervened rats, irrespective of the post-immunisation (Figure 6B,D).

In line with the total IgA in serum, in general total IgA in faeces increased throughout the study, even though statistically significant differences were only observed between naïve rats and at the day of sacrifice for rats intervened with cow's milk and post-immunised with cow's milk and for rats intervened with camel milk and post-immunised with camel milk (Figure S1C,D, Supporting Information). No differences in total IgA in faeces were observed between rats intervened with water and rats intervened with cow's or camel milk at any time point irrespectively of the post-immunisation (Figure 6E-H) except from rats intervened with cow's milk and post-immunised with camel milk (Figure 6G).

Total IgA in SI content at the day of sacrifice did not show significant differences between water, cow's and camel milk intervened rats regardless the post-immunisation (Figure 6I,K), suggesting that neither intervention nor post-immunisation influenced gut mucosal immune homeostasis by changes in IgA.

For rats tested for prevention against CMA, intervention with cow's milk promoted a statistically significant lower level of cow's milk-specific IgA in serum in comparison to rats intervened with water and camel milk (Figure 6J). In contrast, for rats tested for prevention against camel milk allergy, intervention with camel milk did not promote a lower level of camel milk-specific IgA in serum compared to intervention with water or cow's milk, but instead intervention with cow's induced a statistically significantly lower level of camel milk-specific IgA in serum in comparison to rats intervened with camel milk (Figure 6H). This indicates that intervention with cow's milk in general promoted reduced levels of specific IgA.

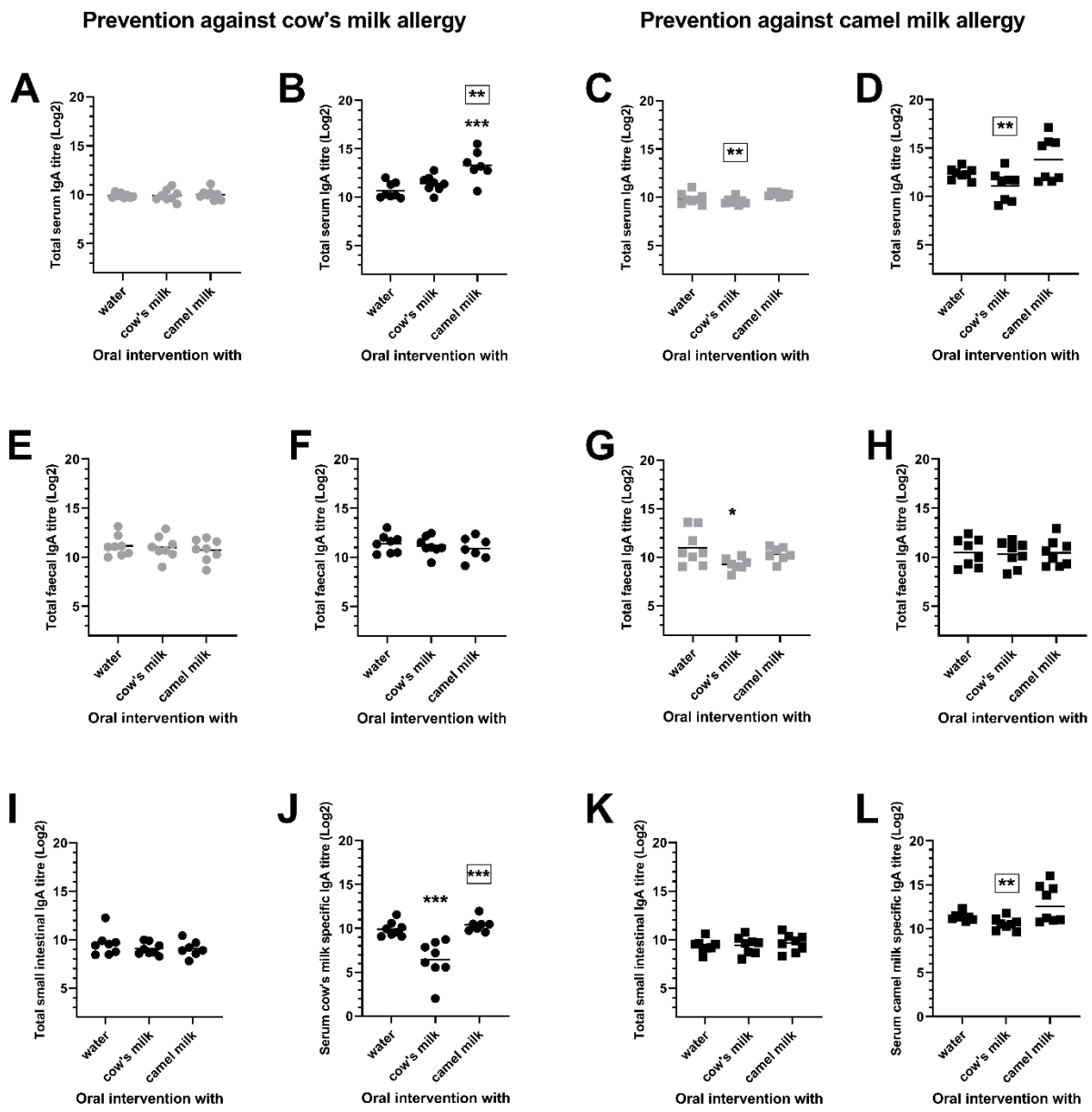

Figure 6. Total and specific IgA. Total IgA in serum **(A)** after intervention phase or **(B)** at sacrifice from rats intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. Total IgA in serum **(C)** after intervention phase or **(D)** at sacrifice from rats intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. Total IgA in faeces **(E)** after intervention phase or **(F)** at sacrifice from rats intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. Total IgA in faeces **(G)** after intervention phase or **(H)** at sacrifice from rats intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. **(I)** Total IgA in small intestine content from rats intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(J)** Serum cow's milk-specific IgA from rats intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy **(K)** Total IgA in small intestine content from rats intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. **(L)** Serum

camel milk-specific IgA from rats intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. Each symbol represents individual rats and horizontal lines indicate mean value. Each color represents different time points of the experiment: (●),(■) represents results after intervention phase while (●),(■) represents results at the day of sacrifice. One-way ANOVA followed by Bonferroni post-test was applied to the water intervened group (unframed) and relative to cow's milk intervened group **(A,B,E,F,I,J)** or to camel milk intervened group **(C,D,G,H,K,L)** (framed). Statistically significant differences are shown as *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001.

3.6. Cellular responses

Cellular immune responses were evaluated by means of gene expression of the Th2 cytokine IL-4, the tolerogenic biomarkers IL-1, CX3XR1, FoxP3, TGF-β and INF-γ, the thigh junction protein Ocln, the epithelial-derived cytokine TSLP and the pro-inflammatory cytokine IL1β in the SI and mLN. In general, no statistically significant differences were observed between any of the intervention groups regardless of whether rats were tested for prevention against cow's or camel milk allergy (Figure 7A-D). Yet, for rats intervened with cow's milk and post-immunised with camel milk a slightly lower expression of CX3XR1 and IL-4 were observed when comparing with water intervened rats (p=0.025 and p=0.0289, respectively, based on Mann-Whitney, Figure 7B).

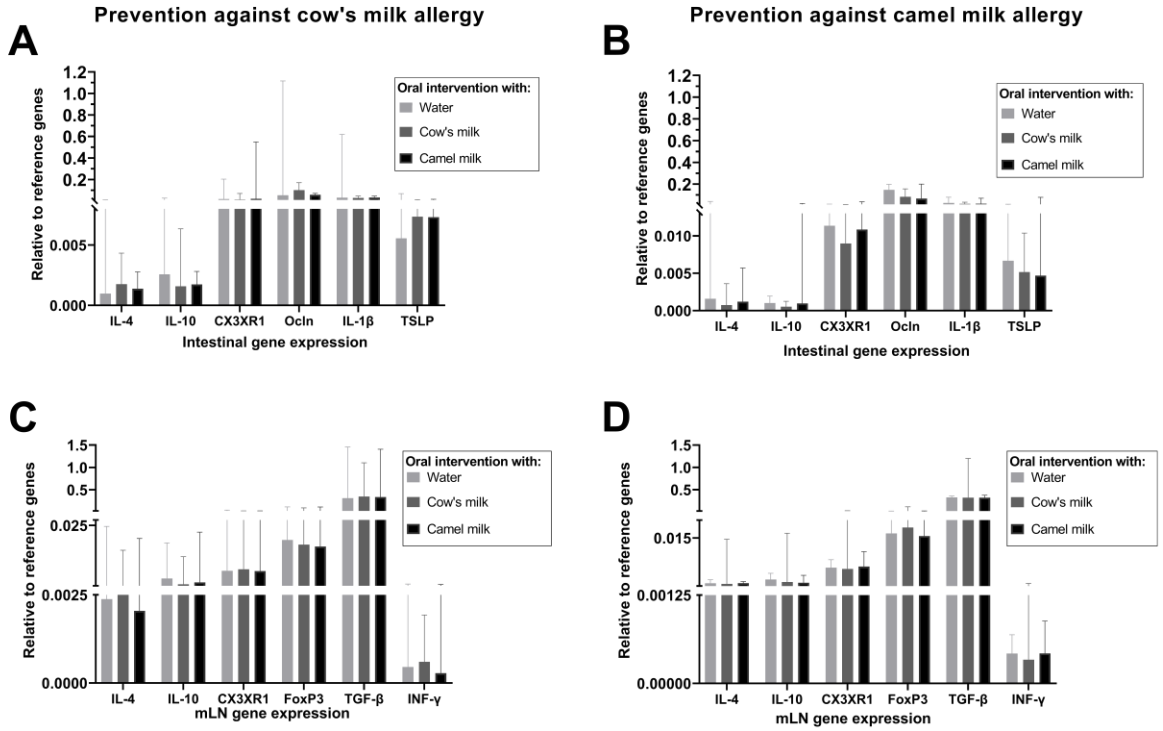

Figure 7. Relative gene expression. Expression of selected genes in the small intestine (SI) from rats intervened with either water, cow's milk or camel milk and tested for prevention against **(A)** cow's milk allergy or **(B)** camel milk allergy. Expression of selected genes in mesenteric lymph nodes (mLN) from rats intervened with either water, cow's milk or camel milk and tested for prevention against **(C)** cow's milk allergy or **(D)** camel milk allergy. Each bar represents the median with 95% confidence interval. Kruskal-

Wallis test followed by Dunn's post-test was applied, where statistical analysis was performed to the water intervened rats and relative to cow's milk **(A,C)** or camel milk **(B,D)** intervened group.

3.7. Intestinal protein uptake

To evaluate intestinal protein uptake in rats intervened with water, cow's and camel milk and tested for prevention against CMA, and thus challenged with cow's milk at the end of the experiment, concentration of BLG as a marker of cow's milk protein uptake, was measured in LP, SI content, EPI and PP. In general, no statistically significant differences were observed in the amount of BLG taken up in each individual intestinal compartment (Figure 8A), even though it was observed that the overall distribution of BLG in the camel milk intervened group differed from the water and cow's milk intervened groups, with a higher proportion of BLG found in LP and PP, and with a consequential lower proportion in SI content (Figure 8B). Interestingly, a small but statistically significant inverse correlation between the amount of BLG in the SI content and total faecal IgA level was observed across all groups, suggesting that the more IgA secreted into the intestinal lumen the less BLG is trapped in the SI content (Figure 8C).

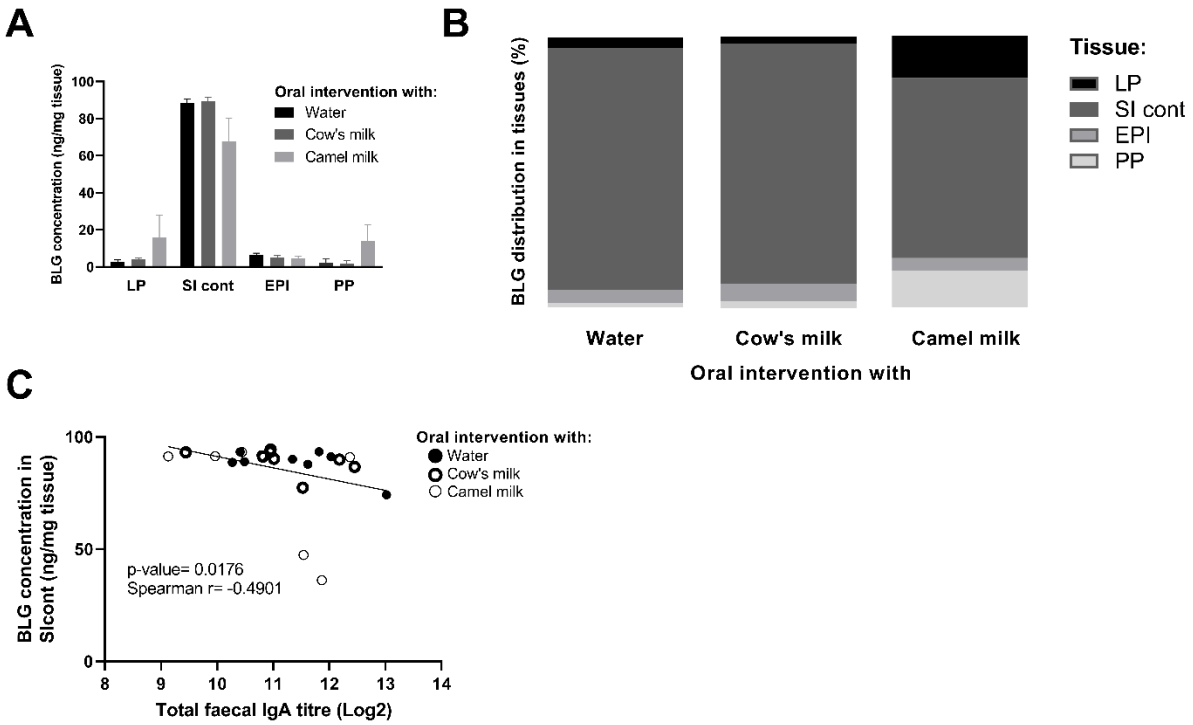

Figure 8. In vivo intestinal protein uptake in rats prevented against cow's milk allergy. (A) β-lactoglobulin (BLG) concentration in ng/mg of tissue. Each bar represents mean value with the standard error for the mean. Kruskal-Wallis test followed by Dunn's post-test was applied. **(B)** Relative distribution of BLG (%) between lamina propria (LP), small intestine content (SI cont), epithelium (EPI) and Peyer's patches (PP) compartments in rats intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(C)** Correlation between total faecal IgA and amount of BLG in SI cont. Non-

parametric Spearman correlations were calculated between all pairs of total faecal IgA and amount of BLG in SI cont.

4. Discussion

Due to lack of solid evidence, it is no longer recommended to use specific infant formulas based on partially hydrolysed cow's milk proteins for prevention of CMA in high-risk infants. At present, there are no specific recommendations for the use of any particular infant formula, but the choice of infant formula should be based on the need of each individual infant [39,80,81,108]. Hence there could be an opportunity for new and alternative infant formulas based on non-hydrolysed proteins.

When designing infant formulas for CMA prevention, it is the delicate balance between inducing a solid oral tolerance still avoiding the development of CMA that should be in focus. Yet, there is limited knowledge on whether the complete repertoire of allergens and epitopes is required to induce a solid tolerance, as previously discussed in several studies investigating the tolerance inducing capacity of hydrolysed cow's milk proteins [86,118,128–130]. Not only may the initial epitope repertoire be different between intact and hydrolysed cow's milk proteins, but peptide fragments may in addition be processed and presented to the gut immune system in a distinct way than their intact counterparts [86]. Consequently, different repertoires of B and T cell epitopes may be presented to the immune system. In line, the same aspect and the possibility of bystander effects have also been discussed for allergy immunotherapy [131], where peptide-based immunotherapy was shown to be effective in some studies [132,133], whereas in other studies it was shown to lack tolerance inducing capacity [134,135]. Thus, it could be speculated that infant formulas based on intact proteins would be more efficient in preventing CMA than those based on hydrolysed proteins as they may cover a larger repertoire of epitopes, containing both linear and conformational epitopes. Yet, this could be on the consequence of their safety.

In the last decade, there has been a growing interest in the usability of milk proteins from other mammalian species for infant formulas [7]. Whereas goat and sheep milk have been shown to cause allergic reactions in cow's milk allergic patients, probably due to the high homology of goat and sheep milk proteins with cow's milk proteins [8,9,105], donkey, horse and camel milk have in general been shown to be safer for cow's milk allergic patients, probably due to the lower homology with cow's milk proteins [8,24,102,106]. Yet, none of them have been investigated for their potential in CMA prevention. In this study, we focused on camel milk and evaluated whether it could prevent CMA and whether cow's milk could prevent camel milk allergy using a well-established prophylactic BN rat model [98,116,118].

We demonstrated that neither camel milk nor cow's milk induced sensitisation upon three weeks of *ad libitum* administration. Yet, both camel and cow's milk were shown to induce specific IgG1 upon three weeks of *ad libitum* administration, indicating that they were easily recognised by the immune system, which is a prerequisite for oral tolerance induction [98]. Moreover, in line with human studies [8,21,25,124,125], as well as in our previous i.p. sensitisation study [21], a low cross-reactivity between cow's and camel milk proteins was revealed.

Results showed that whereas cow's milk was efficient in preventing cow's milk sensitisation, camel milk was not capable of preventing sensitisation to cow's milk. Similarly, camel milk was efficient in preventing camel milk sensitisation. Interesting, cow's milk was shown to have a small though transient capacity to prevent sensitisation to camel milk. Similar patterns were demonstrated when evaluating the clinically relevance of the sensitisation to cow's and camel milk, where cow's milk could prevent a clinically relevant CMA, and camel milk could easily prevent a clinically relevant camel milk allergy. Contrary, camel milk could not prevent clinical symptoms of cow's milk allergy, whereas cow's milk had a small effect on the clinical manifestation of camel milk allergy. These results were well aligned with immunoblotting results, showing no differences in the pattern of the presence and intensity of IgG1 reactive proteins between groups intervened with water and groups intervened with cow's and camel milk, when prevention was tested against camel and cow's milk allergy, respectively.

Overall, our results showed a low cross-tolerogenic capacity between cow's and camel milk proteins, probably due to their low protein homology, where protein sequence alignments demonstrate protein identities of 47-81% [21]. Thus, the overlapping epitope repertoire between cow's and camel milk proteins seemed too low to provide solid cross-prevention, and hence this study did not provide evidence for a bystander effect of co-existing epitopes. Collectively, this stresses that camel milk would not be a suitable protein source for infant formulas for CMA prevention. We hypothesise that donkey and horse milk proteins due to even lower protein homologies with cow's milk proteins, with protein sequence identities of 46-74% [7] would likewise not provide a suitable source for CMA prevention, whereas goat and sheep milk proteins, with protein sequence identifies to cow's milk proteins of 85-95% [7] would probably be a better option. It has been suggested that a high degree of homology is needed between proteins in order to obtain a bystander effect and drive a tolerance towards counterpart proteins [136]. For example, in several studies it was shown that birch pollen immunotherapy only had limited effect on the related apple allergy [137–139], due to too low homology between the main allergen in birch pollen and apple i.e. Bet v 1 and Mal d 1 [140]. Contrary, in a study by Elizur et al., it was shown that cashew oral immunotherapy was not only efficient in inducing tolerance to cashew, but also to pistachio [141], probably due to a general higher protein sequence homology between major allergens [142].

Even though it was low and transient, this study demonstrated some cross-tolerance inducing capacity of cow's milk against camel milk allergy. We hypothesise that the reason for cow's milk containing some cross-preventive capacity in contrast to camel milk, is due to the fact that cow's milk contains all major milk allergens [143], whereas camel milk do not contain BLG. Consequently, camel milk may not be capable of inducing tolerance against BLG, which is one of the major allergens found in cow's milk for which it is reported that up to 80% of cow's milk allergic patients have specific IgE against [144]. In contrast to camel milk, milk from goat, sheep,

donkey and horse contain BLG [145], and thus may be able to drive tolerance towards all cow's milk allergens.

Cow's and camel milk were both shown to have some immunomodulatory effects, yet they were shown to be distinct and seemed to be independent of their allergy preventive capacity. While cow's milk seemed to restrain immune responses, reflected in lower specific IgA levels and a slightly lower expression of intestinal IL-4 and CX3CR1, camel milk seemed to have an immune stimulatory capacity, as camel milk promoted total serum IgA. Strong immunomodulatory properties of camel milk has also previously been suggested [146].

In conclusion, this study demonstrated a low cross-tolerogenic capacity of camel and cow's milk proteins, indicating that camel milk is not a good candidate as a protein source for infant formulas in CMA prevention. For CMA prevention proteins from other mammalian milk such as goat and sheep, having higher protein amino acid sequence identities to counterpart proteins in cow's milk as well as containing BLG, may provide a more suitable solutions for CMA prevention.

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Author contributions

Conceptualisation: NZM and KLB. Investigation and data curation: NZM, MHS and KLB. Methodology: NZM, MHS, ASRB, AIS and KLB. Data visualisation: NZM, MHS and KLB. Formal Analysis: NZM, MHS and KLB. Supervision: KLB. Writing – original draft: NZM. Writing – review & editing: NZM, MHS, ASRB, AIS, EBH and KLB. All authors made substantial intellectual contributions to the study, reviewed the manuscript critically, and approved the final version of the manuscript.

7 Manuscript VI

Reactivity towards camel milk products in cow's milk allergic children

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Study report.

Reactivity towards camel milk products in cow's milk allergic children

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Abstract

Introduction: The most common type of food allergy among infants and children is cow's milk allergy (CMA) and it affects 0.5-3.8% of them worldwide. CMA management in non-exclusively breastfed infants requires the use of hypoallergenic infant formulas based on extensively hydrolysed cow's milk proteins. Mammalian milks such as horse, donkey and camel milk have recently gained an interest for their utility as protein sources in potential, alternative infant formulas for CMA management. Several studies showed that camel milk proteins have a low cross-reactivity with cow's milk proteins, yet some individuals still reacted to camel milk. Processing such as enzyme hydrolysis and heat treatment could potentially even further increase tolerance to camel milk proteins in children with CMA.

Methods: Sera from children with CMA (n=9) were analysed for IgG and IgE reactivity towards intact, EH and HT cow's and camel milk products using ELISAs. Further basophil activation test (BAT) was performed using blood samples from children with CMA (n=3) to evaluate basophil activation using intact, EH and HT cow's and camel milk products.

Results: There was a decreased IgG and IgE reactivity towards EH cow's milk but not HT cow's milk compared to intact cow's milk. Decreased reactivity of IgG and IgE was also measured for all camel milk products, being highest for camel milk and decreased further for HT camel milk and EH camel milk respectively.

Conclusions: This study showed that cow's and camel milk had a low cross-reactivity in children with CMA which could be even decreased by processing methods such as enzyme hydrolysis and perhaps heat treatment.

1. Introduction

The most common type of food allergy among infants and children is IgE-mediated cow's milk allergy (CMA) and it affects 0.5-3.8% of them worldwide [5,71,147]. For infants up to 6 months of age, breastfeeding is the first feeding option suggested [148], and if an infant suffers from CMA, elimination of cow's milk proteins from mothers' diet is recommended [149]. However, if breastfeeding is not possible and an infant suffers from CMA, the use of hypoallergenic infant formula is needed [38,150]. Hypoallergenic infant formulas are based on extensively hydrolysed cow's milk proteins for their allergenicity reduction [38]. The majority of infants with CMA tolerate extensively hydrolysed formulas (eHFs). Yet, for infants who still manifest clinical symptoms after use of eHF, there is a need to use an amino acid-based formula [151].

Camel milk has recently gained an interest for its usefulness in CMA management [24,152,153]. It is mostly since camel milk lacks β-lactoglobulin (BLG) [23], one of the main allergen found in cow's milk and also due to its low homology with cow's milk proteins [21]. Several studies analysing blood samples from children with CMA showed low or no cross-reactivity between cow's and camel milk proteins [8,25,125,152]. In addition, in studies where skin prick test (SPT) was used, most children with CMA did not show any reaction to camel milk, however, there were single cases with positive SPT [26,124].

Enzyme hydrolysis is a commonly used processing method for cow's milk proteins allergenicity reduction for hypoallergenic infant formula production. Yet, heat treatment has recently gained an interest as a processing method to be potentially used for proteins allergenicity reduction [49]. There is some evidence showing that baked milk was well tolerated in individuals with CMA [154] and that baked milk can accelerate induction of tolerance to intact cow's milk proteins [155,156]. On the other hand, Abbring S. et al. showed that allergenicity of cow's milk proteins was increased by heat treatment [97]. Such processing methods could be applied to camel milk to alter its protein structures, potentially improving its tolerance in children with CMA who manifested a reaction to camel milk proteins.

The knowledge on how enzyme hydrolysis and heat treatment influence cross-reactivity of cow's and camel milk proteins has to our knowledge not yet been investigated. In the present study, we evaluated how enzyme hydrolysis and heat treatment influenced cross-reactivity between cow's and camel milk proteins in children with CMA using enzyme-linked immunosorbent assays (ELISAs) for IgG and IgE detection. Moreover, using a basophil activation test (BAT), this study evaluated whether intact, enzyme hydrolysed (EH) and heat treated (HT) camel milk could induce basophil activation in individuals with CMA.

2. Materials and Methods

2.1. Materials

Cow's milk powder from MlekPol, Grajewo, Poland was purchased in a local Polish shop. Camel milk powder was kindly provided by Ausnutria Dairy (China) Co., Ltd, (Changsha, Hunan, China). Powders were dissolved in phosphate buffer saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) to obtain milk products in solution.

2.1.1. Processed cow's and camel milk products

Enzyme hydrolysis of cow's and camel milk was performed as described in **Manuscript III**. Briefly, 50 mg/mL of cow's and camel milk proteins were hydrolysed with Pancreatic Trypsin Novo 6.0 S, Type Salt Free (Trp) (Novozymes, Bagsværd, Denmark) and Alcalase 2.4 L FG (Alc) (Novozymes). To obtain HT cow's and camel milk, 50 mg/mL of cow's and camel milk proteins were used and heat treatment was applied by means of 121 ^{o}C for 1h.

2.1.2. Human samples

Plasma or serum samples were collected at the Medical University of Vienna, Vienna, Austria from children with confirmed CMA by either food challenge or a clear history of a clinical reaction upon proven exposure to cow's milk. Nine serum/plasma samples were used to assess specific antibody levels while three blood samples were used for the basophil activation test (BAT). The study protocol was approved by the ethics board of the Medical University of Vienna (EK Nr-1852/2017). All participants and their guardians gave written informed consent.

2.2. Indirect ELISA for IgG detection

To detect IgG specific for milk products, indirect ELISAs were performed using Maxisorp microtitre plates (96-well, Nunc, Roskilde, Denmark). Plates were coated with 100 µL/well of 10 µg/mL of intact cow's milk, intact camel milk, HT cow's milk, HT camel milk, EH cow's milk, or EH camel milk, in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated at 4 °C overnight. Plates were washed five times with PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) with 0.01% (w/v) Tween 20 (PBS-T) between each step. For all steps, plates were incubated at RT for 1 h in the dark with gentle agitation. First, plates were incubated with 50 µL/well of two-fold serial dilution of serum or plasma samples (v/v) in PBS-T. Further, 50 µL/well of secondary antibody (horseradish peroxidase (HRP)-labelled-mouse-anti-human IgG Fc-HRP, 9042-05, Southern Biotech, Birmingham, AL, USA) diluted 1:8,000 (v/v) in PBS-T was added to the plates. After incubation, plates were additionally washed twice with tap water and incubated with 100 µL/well of TMB-one (Kementec Diagnosis, Taastrup, Denmark) for 12 min. The reaction was stopped with 100 µL/well of 0.2 M H_2SO_4 and the absorbance was measured. The results were expressed as log2 titre value with an individual cut-off values to an optical density (OD) determined individually for each product.

2.3. Antibody Capture ELISA for specific IgE detection

To detect IgE specific for intact cow's milk, intact camel milk, HT cow's milk, HT camel milk, EH cow's milk, and EH camel milk, antibody capture ELISAs were performed using Maxisorp microtitre plates (96-well, Nunc) coated with 100 µL/well of goat anti-human IgE (A18795, Thermo Fisher Scientific, Waltham, MA, US) diluted 1:2,000 in coating buffer and incubated at 4 °C overnight. Plates were washed five times with PBS-T between each step. For all steps, plates were incubated at RT for one hour in the dark with gentle agitation, except the blocking step where plates were incubated in 37 °C without agitation. For intact cow's milk and intact camel milk specific IgE detection, plates were blocked with 5% (v/v) horse serum diluted in PBS-T, for HT cow's milk, HT camel milk and EH cow's milk specific IgE detection, plates were blocked with 10% (v/v) rabbit serum diluted in PBS-T, and for EH camel milk specific IgE detection, plates were blocked with 5% (v/v) rabbit serum diluted in PBS-T. Subsequently, plates were incubated for one hour with 50 µL/well of two-fold serial dilution of serum or plasma samples (v/v) in the blocking solution. Further, 50 µL/well of 1:1,000 of digoxigenin (DIG)-coupled intact cow's milk and intact camel milk in PBS-T, 1:100 of DIG-coupled HT cow's milk, HT camel milk, and EH cow's milk in 10% (v/v) rabbit serum, and 1:50 of DIG-coupled EH camel milk in 5% (v/v) rabbit serum were added. Finally, for all products, plates were incubated with 100 µL/well of HRP-labelled sheep-anti-DIG-POD (11633716001, Roche, Diagnostics GmbH, Mannheim, Germany) diluted 1:1,000 (v/v) in PBS-T. Plates were additionally washed twice with tap water and incubated with 100 µL/well of TMB-one (Kementec Diagnosis) for 12 min. The reaction was stopped with 100 µL/well of 0.2 M H_2SO_4 and the absorbance was measured. The results were expressed as log2 titre values with individual cut-off values to an OD determined individually for each product.

2.4. Basophil activation test (BAT)

To evaluate whether intact cow's milk, intact camel milk, HT cow's milk, HT camel milk, EH cow's milk, and EH camel milk activate basophils of individuals with CMA, the upregulation of CD63 on the surface of basophils was measured upon stimulation with the above-mentioned milk products by flow cytometry following the manufacturer's instructions (FlowCAST Buehlmann, Switzerland). In brief, heparinised whole blood was stimulated in a serial 10-fold dilution (1-10000 ng/mL) with each of the milk products in the presence of fluorescence antibodies to CD63 and the chemokine receptor CCR3. A specific monoclonal antibody binding to the high affinity IgE receptor (FcεRI) and the unspecific cell activator fMLP were used as positive controls for degranulation. Basophils were defined as $SSC^{low}/CCR3^{+}$ cell population and activation was measured as the percentage of CD63+ basophils compared to non-stimulated cells. All flow cytometry experiments were performed with a BD FACS Canto II (Becton, Dickinson and Company, NJ, US).

2.5. Statistics

Graphs and if applicable statistical analyses were performed using GraphPrism version 9.0.1 (San Diego, CA, USA). Results from ELISAs were expressed as log2 titre values. Only results from indirect ELISAs were subjected to statistical analyses where D'Agostino-Pearson normality test was first performed. Differences between more than two groups were analysed using Kruskal-Wallis test followed by Dunn's post-test for multiple comparison. Differences between the products specific IgG were regarded as statistically significant if $P \leq 0.05$. Asterisks indicate statistically significant differences between two given groups: $*$ = $P \leq 0.05$, $**$ = $P \leq 0.01$, $***$ = $P \leq 0.001$, $****$ $P \leq 0.0001$ and ns indicate no statistically significant.

3. Results

3.1. Processing decreased cross-reactivity between cow's and camel milk proteins

To evaluate the pattern of IgG reactivity in serum/plasma samples from children with CMA, the level of IgG binding towards intact cow's milk, intact camel milk, HT cow's milk, HT camel milk, EH cow's milk and EH camel milk was measured and expressed in titre values. There was no significant difference between the IgG binding towards cow's milk and HT cow's milk, but the antibody binding towards EH cow's milk was significantly lower when compared with intact cow's milk (Figure 1A). This indicates that while enzyme hydrolysis of cow's milk proteins reduced their immune recognition in individuals with CMA, heat treatment of cow's milk proteins did not change their immune binding. Similarly, there was no significant difference between the IgG binding towards intact camel milk and HT camel milk, but the antibody binding towards EH camel milk was significantly lower when comparing with intact camel milk (Figure 1A). Yet, figure 1A clearly shows that IgG antibody binding was lower for all camel milk products when comparing with cow's milk. Even though, no significant difference was observed between IgG binding to cow's milk and camel milk, there was four times less binding to intact camel milk when compared to intact cow's milk (Figure 1A).

In addition to evaluate whether heat treatment and enzyme hydrolysis reduce cross-reactivity between cow's and camel milk proteins, as well as whether heat treatment and enzyme hydrolysis reduce allergenicity of cow's milk, the level of IgE binding was measured, as IgE is the key player in IgE mediated food allergy [157]. While heat treatment did not reduce the allergenicity of cow's milk proteins as all children showed IgE reactivity towards HT cow's milk, enzyme hydrolysis reduced cow's milk allergenicity as only 3 children showed IgE reactivity towards EH cow's milk (Figure 1B). In addition, only some children raised IgE that reacted with intact camel milk and HT camel milk indicating a low cross-reactivity between cow's and camel milk proteins being further decreased with enzyme hydrolysis of camel milk as only one child raised IgE that reacted with EH camel milk as shown in Figure 1B.

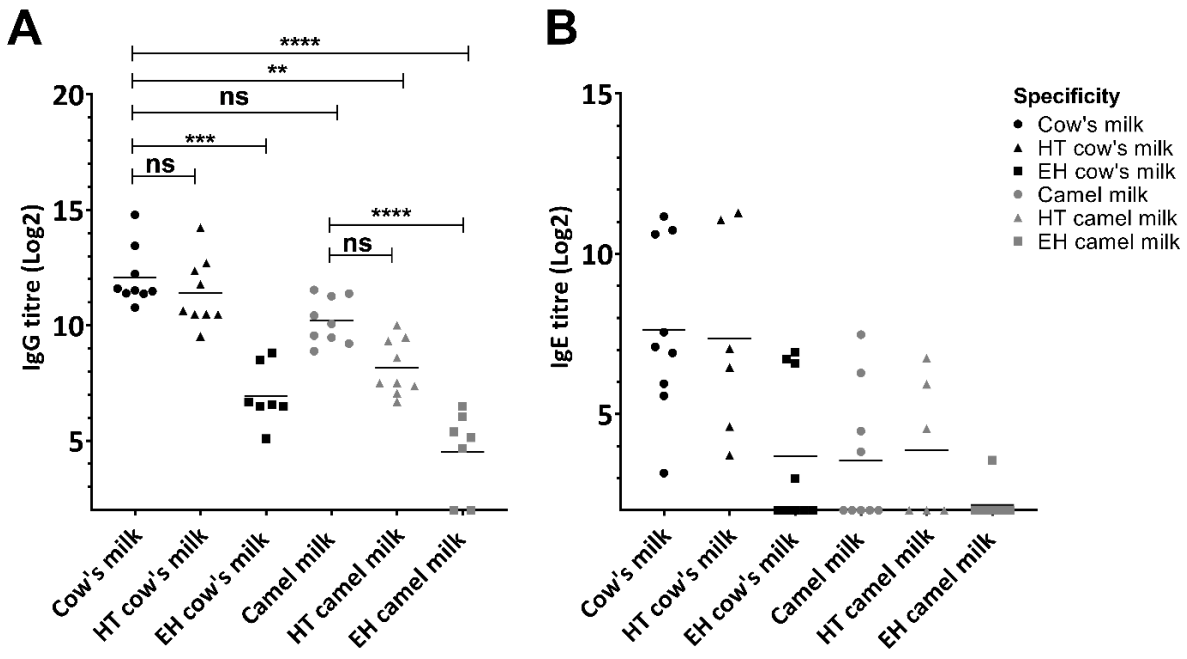

Figure 1. Reactivity of IgG and IgE antibodies in individuals with cow's milk allergy (CMA) towards: (●) intact cow's milk, (■) EH cow's milk, (▲) HT cow's milk, (●) intact camel milk, (■) EH camel milk and (▲) HT camel milk. **(A)** Comparison of IgG reactivity. Statistically significant differences in reactivity between intact cow's and camel milk and their HT and EH versions as well as in reactivity towards cow's milk and all milk products, were analysed using Kruskal-Wallis test followed by Dunn's post-test for multiple comparison. Asterisks indicates statistically significant differences as *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ∗ ∗∗∗ P ≤ 0.0001 and ns, no statistically significant. **(B)** Comparison of IgE reactivity. Each symbol represents an individual sample. Horizontal lines on the graph display the median **(A)** and mean **(B)** values for each product.

3.2. Basophil activation test

In order to evaluate ex vivo basophil activation in a limited number of blood samples from children with CMA (n=3), BAT tests were performed to determine the upregulation of CD63 on the surface of basophils after their stimulation with different cow's and camel milk products. Figure 2A, 2B, and 2C displays results from individual children after stimulation with intact cow's milk, intact camel milk, HT cow's milk, HT camel milk, EH cow's milk, and EH camel milk. Due to the sample limitation, one sample (Figure 2A) was only stimulated with cow's milk, camel milk, HT cow's milk, and HT camel milk. In sample A, intact cow's milk and HT cow's milk caused approx. 80% of basophil activation already at the concentration of 100 ng/mL (Figure 2A). On the other hand, intact camel milk and HT camel milk activated approx. 60% of basophils and at a maximum concentration i.e. 10000 ng/mL (Figure 2A). Further, in samples B and C approx. 20% and 40% of basophils respectively were activated at the highest concentrations of intact cow's milk while other products were not able to activate basophils in those samples at all except HT cow's milk

which activated approx. 10% of basophils in sample C at the highest concentration (Figure 2B and 2C).

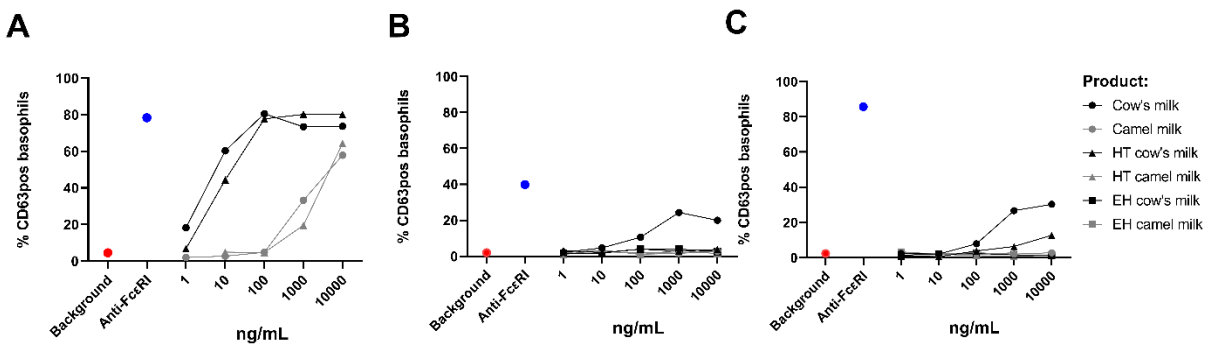

Figure 2. Basophil activation test. Ex vivo basophil activation performed with whole blood of individuals with cow's milk allergy (CMA) basophils expressing CD63 activation markers. The results are presented as %CD63 positive basophils at serial dilutions from 1 ng/mL to 10000 ng/mL of different milk products: (●) intact cow's milk, (■) EH cow's milk, (▲) HT cow's milk, (●) intact camel milk, (■) EH camel milk and (▲) HT camel milk. ● represents individual sample background and ● represents positive control (Anti-FceRI). Each graph **(A), (B), (C)** represents the results of an individual sample.

4. Discussion

Camel milk has recently gained an interest for its applicability in CMA management due to the lack of BLG and low homology with cow's milk proteins [21,23]. While processing to reduce cow's milk proteins allergenicity has been well studied [28,48,92], to our knowledge no studies were conducted yet where the processing of camel milk and its influence on cross-reactivity with cow's milk proteins was evaluated. In this study, it was demonstrated that processing such as enzyme hydrolysis and probably heat treatment reduced cross-reactivity between cow's and camel milk proteins in children with CMA.

Certainly, parameters of processing such as temperature and time for heat treatment as well as enzyme specificity and duration of hydrolysis for enzyme hydrolysis are crucial for their influence on the degree of proteins modification [27,46,49]. In the present study, we showed that heat treatment of cow's milk did not reduce its allergenicity which was in line with Abbring et al. who suggested that heat treatment has no positive effect on cow's milk proteins allergenicity reduction [97]. In the contrary, Nowak-Wegrzyn et al. showed that extensively heat treated cow's milk was well tolerated in children with CMA [95]. Moreover, baked milk was shown to accelerate the resolution of cow's milk allergy [155,156]. This clearly shows that differences in heat treatment parameters as well as matrix effect play an important role in whether allergenicity of cow's milk proteins is reduced or even increased [46,93,158]. Based on the results in **Manuscript III**, it was shown that HT cow's milk contains much more aggregated proteins as well as increased number of amino-acid cross-links caused by heat treatment, when compared with intact cow's milk. As the allergenicity of HT cow's milk was not reduced, it could be speculated that still a great amount of denatured but not aggregated whey proteins was available, where their unfolding

caused exposure of hidden allergic epitopes or that neo epitopes on the surface of aggregates were created [159]. For HT camel milk, there is no equivocal conclusion on whether heat treatment of camel milk could even decrease its cross-reactivity with cow's milk proteins. In this study, out of three children who showed cross-reactivity with HT camel milk, two of them had a slightly lower IgE titre value when compared with the IgE titre value for intact camel milk. Indeed an analysis using a higher number of samples would be of a high benefit to make more clear conclusions.

Based on the results in **Manuscript III** it was shown that no intact proteins were left after EH of cow's and camel milk. Both EH cow's and camel milk however contained peptides of around 5 kDa thus equal to an average length of 45 aa. In this study only one child cross-react with EH camel milk. Therefore, partial hydrolysis of camel milk seems to be a good option to improve camel milk usefulness for those individuals who still showed cross-reactivity with intact camel milk.

BAT is an important diagnostic tool used as a clinical marker also in CMA [160]. In this study using a limited number of blood samples from children with CMA, BAT with different cow's and camel milk products was performed. Even though BAT was only performed using blood samples from three children, it clearly showed that activation of basophils differed between different patients and different milk products that basophils were stimulated with. The differences were observed in basophil activation caused by intact cow's milk where in the first sample approx. 80% were activated while in the other two samples only 20-40% activation was achieved. The differences observed could be explained by different specific IgE level as well as differences in observed symptoms during challenge with cow's milk, suggesting that the higher level of specific IgE and symptoms, the more basophils activated [161]. Intact camel milk as well as HT camel milk triggered an equally high basophil activation but only for sample A. For the second and third sample, probably due to the low activation with intact cow's milk, other milk products did not show any activation at all. These results, showing a great heterogenicity between different patients are in line with Morisawa et al., where using histamine release assay it was indicated that ten patients showed very different patterns of histamine release upon stimulation with cow's milk, HT cow's milk and EH cow's milk [162]. Performing BAT test with a higher number of blood samples from children with severe CMA, using all milk products used in this study would be of a great value for further evaluation of camel milk products usefulness in CMA management.

5. Conclusions

This study showed that intact cow's and camel milk had a low cross-reactivity in children with CMA which could be even decreased by processing methods such as enzyme hydrolysis and perhaps heat treatment.

8 General discussion

Cow's milk allergy (CMA) is the most common type of food allergy among infants and small children [5]. CMA management is based on selection of appropriate hypoallergenic infant formula to suit individual needs [108], when breastfeeding is not an option. For CMA prevention, at present, there are no specific recommendations for the use of any particular infant formula but as for CMA management a choice should be matched to individual needs [39].
Increasing interest in alternative mammalian milks, increases a need for their overall sensitising and capacity evaluation and also detailed evaluation of their potency to be used in CMA management and prevention. Camel milk is the main focus in this PhD project, as an alternative source of proteins in infant formula manufacture. Therefore, the aims of this project were to evaluate immunogenicity, sensitising, cross-reactive and tolerogenic capacity of camel milk in order to obtain solid scientific evidences for its usefulness in CMA management and prevention.

8.1. Allergenicity of camel milk

In this PhD project allergenicity of camel milk using BN rat model was evaluated for the first time showing that camel milk was as good to induce sensitisation as cow's milk (**Manuscript II** and **Manuscript IV**). From the perspective of camel milk allergenicity in a human population, there is almost no data available. This is due to the fact that camel milk is in general much less available worldwide as it only corresponds to 0.4% of the global milk production [16] . Allergy to camel milk may be increased in countries where camel milk is easily available such as Middle East, parts of Africa, Asia and Australia, though not yet well evaluated. At present there is one human study available where allergy to camel milk was confirmed in nine patients [163], as well as one case report where anaphylaxis to camel milk was reported in an atopic child [164]. In this PhD project, results showed that cow's milk could be an alternative in individuals with camel milk allergy due to their low cross-reactivity. However, it should be highlighted that cow's milk contains β-lactoglobulin (BLG) that is not present in camel milk [23], and is also one of the most allergenic proteins in cow's milk [165]. Therefore when introducing cow's milk in individual with camel milk allergy, additional allergy towards BLG may be induced.
Enzyme hydrolysis and heat treatment were evaluated as a processing methods to reduce allergenicity of camel milk proteins. Results in **Manuscript IV** showed that enzyme hydrolysis is an effective method to reduce allergenicity of camel milk proteins. Heat treatment used in this PhD project showed not to reduce allergenicity of camel milk but it partially did for cow's milk. This is due to the fact that cow's and camel milk because of their different proteins and other components composition, behaved differently under heat treatment as shown in **Manuscript III**. This is very important knowledge for the future application of processing methods such as enzyme hydrolysis and heat treatment to modify camel milk proteins, highlighting that in order

to obtain comparable degree of proteins modifications in cow's and camel milk, processing parameters should be applied individually.

8.2. Tolerogenic properties of camel milk

In this PhD project tolerogenic capacity of camel milk using prophylactic BN rat model was evaluated for the first time showing that oral introduction of camel milk did not induce any sensitisation, thus is a good prerequisite for oral tolerance induction (**Manuscript V**). The same was observed for cow's milk. However, proteins in camel milk could not drive tolerance to counterpart proteins in cow's milk probably due to their low sequence homology [21]. On the other hand, in a study by Levy et al. it was observed that patients allergic to cow's milk who showed cross-reactivity with goat and sheep milk proteins, after oral immunotherapy (OIT) with cow's milk and successful tolerance induction, they also showed induced tolerance to goat and sheep milk proteins [166]. This means that probably due to the high sequence homology between cow's milk and goat and sheep milk proteins [21], induction of tolerance to proteins in cow's milk, could drive tolerance to proteins in goat and sheep milk.

8.3. Camel milk in infant formula production

There are two protein fractions in milk that are used in infant formula production i.e. whey and caseins, with their ratio adjusted or used separately [167]. In **Manuscript II,** it was shown that there was a lower cross-reactivity between cow's and camel milk caseins than whey proteins. This knowledge could be used for even better utility of camel milk in the production of infant formula. Selection of the best suited infant formula for an individual needs is crucial for the best CMA management. For example, if an infant with CMA raised mainly IgEs specific for BLG, use of whole camel milk based infant formula could be a good choice. However if reactivity to other whey proteins is detected, especially towards SA that in **Manuscript II** and **Manuscript IV** showed to be the most cross-reactive cow's and camel milk protein, use of camel milk casein based infant formula would be a better choice.

In this PhD project it was shown that processing such as enzyme hydrolysis could be applied in camel milk for proteins partial digestion as in **Manuscript IV** it was shown that enzyme hydrolysis reduced sensitising and cross-reactive capacity of camel milk still keeping its immunogenicity which is a good feature of potential tolerance induction. In **Manuscript VI** enzyme hydrolysis showed to reduce cow's and camel milk proteins cross-reactivity, indicating that enzyme hydrolysed camel milk could be used in individuals with CMA who showed a reactivity towards intact camel milk.

Camel milk contains approx. three times more vitamin C when comparing with cow's milk [168]. Moreover, camel milk was evaluated for its beneficial immunomodulatory components and bioactive peptides [59,123,146,169], which could also be of a huge value when producing camel milk based infant formulas.

9 Conclusion

The results from this PhD project showed that camel milk is a potential alternative to hypoallergenic infant formulas based on hydrolysed cow's milk proteins used in CMA management for most of the infants and small children with CMA. In addition, partial hydrolysis and heat treatment of camel milk showed to be efficient methods to improve usefulness of camel milk in CMA management.

The results from this PhD project showed that camel milk could not be used for CMA prevention. In general this emphasizes that when one product is suitable for particular allergy management, there is a high chance it will not be suitable for its prevention.

In order to design infant formula based on camel milk proteins with the aim to manage CMA, further human studies with infants and small children allergic to cow's milk are needed.

References

1. Sabaté J, Soret S. Sustainability of plant-based diets: Back to the future. Am J Clin Nutr. 2014;100:476–82.

2. Global Plant Based Food Market Report 2020-2027: Rising Industry Concentration with Growth in Mergers and Acquisitions in the Plant-Based Products Space [Internet]. Available from: https://www.prnewswire.com/news-releases/global-plant-based-food-market-report-2020-2027-rising-industry-concentration-with-growth-in-mergers-and-acquisitions-in-the-plant-based-products-space-301268737.html

3. Khalesi M, Salami M, Moslehishad M, Winterburn J, Moosavi-Movahedi AA. Biomolecular content of camel milk: A traditional superfood towards future healthcare industry. Trends Food Sci Technol. 2017;62:49–58.

4. Faye B, Konuspayeva G. The sustainability challenge to the dairy sector - The growing importance of non-cattle milk production worldwide. Int Dairy J. 2012;24:50–6.

5. Flom JD, Sicherer SH. Epidemiology of cow's milk allergy. Nutrients. 2019;11.

6. Fiocchi A, Bognanni A, Brożek J, Ebisawa M, Schünemann H, Ansotegui IJ, Arasi S, Assa'ad AH, Bahna SL, Canani RB, et al. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines update – I – Plan and definitions. World Allergy Organ J. 2022;15.

7. Maryniak NZ, Sancho AI, Hansen EB, Bøgh KL. Alternatives to Cow's Milk - Based Infant Formulas in the Prevention and Management of Cow's Milk Allergy. Foods. 2022;11.

8. Restani P, Beretta B, Fiocchi A, Ballabio C, Galli CL. Cross-reactivity between mammalian proteins. Ann Allergy, Asthma Immunol. 2002;89:11–5.

9. Infante Pina D, Tormo Carnice R, Conde Zandueta M. Use of goat's milk in patients with cow's milk allergy. An Pediatr. 2003;59:138–42.

10. Vojdani A, Turnpaugh C, Vojdani E. Immune reactivity against a variety of mammalian milks and plant-based milk substitutes. J Dairy Res. 2018;85:358–65.

11. Faye B. The camel, new challenges for a sustainable development. Trop Anim Health Prod. 2016;48:689–92.

12. Meyer K, Hummel J, Clauss M. The relationship between forage cell wall content and voluntary food intake in mammalian herbivores. Mamm Rev. 2010;40:221–45.

13. Dittmann MT, Runge U, Lang RA, Moser D, Galeffi C, Kreuzer M, Clauss M. Methane emission by camelids. PLoS One. 2014;9.

14. Zou Z, Duley JA, Cowley DM, Reed S, Arachchige BJ, Shaw PN, Bansal N. Comprehensive biochemical and proteomic characterization of seasonal Australian camel milk. Food Chem. 2022;381.

15. Berhe T, Seifu E, Ipsen R, Kurtu MY, Hansen EB. Processing Challenges and Opportunities of Camel Dairy Products. Int J Food Sci. 2017;2017.

16. Roy D, Ye A, Moughan PJ, Singh H. Composition, Structure, and Digestive Dynamics of Milk From Different Species—A Review. Front Nutr. 2020;7:1–17.

17. Genene A, Hansen EB, Eshetu M, Hailu Y, Ipsen R. Effect of heat treatment on denaturation of whey protein and resultant rennetability of camel milk. Lwt. 2019;101:404–9.

18. Singh R, Mal G, Kumar D, Patil N V., Pathak KML. Camel Milk: An Important Natural Adjuvant. Agric Res. 2017;6:327–40.

19. Mudgil P, Baba WN, Alneyadi M, Ali Redha A, Maqsood S. Production, characterization, and bioactivity of novel camel milk-based infant formula in comparison to bovine and commercial sources. Lwt. 2021;154:112813.

20. Mudgil P, Baba WN, Alneyadi M, Ali Redha A, Maqsood S. Production, characterization, and bioactivity of novel camel milk-based infant formula in comparison to bovine and commercial sources. Lwt. 2022;154.

21. Maryniak NZ, Hansen EB, Ballegaard A-SR, Sancho AI, Bøgh KL. Comparison of the allergenicity and immunogenicity of Camel and cow's milk—A study in brown Norway rats. Nutrients. 2018;10.

22. Hailu Y, Hansen EB, Seifu E, Eshetu M, Ipsen R, Kappeler S. Functional and technological properties of camel milk proteins: a review. J Dairy Res. 2016;83:422–9.

23. Omar A, Harbourne N, Oruna-Concha MJ. Quantification of major camel milk proteins by capillary electrophoresis. Int Dairy J. 2016;58:31–5.

24. Navarrete-Rodríguez EM, Ríos-Villalobos LA, Alcocer-Arreguín CR, Del-Rio-Navarro BE, Del Rio-Chivardi JM, Saucedo-Ramírez OJ, Sienra-Monge JJL, Frias R V. Cross-over clinical trial for evaluating the safety of camel's milk intake in patients who are allergic to cow's milk protein. Allergol Imunopathologia. 2017;46:149–154.

25. El-Agamy EI, Nawar M, Shamsia SM, Awad S, Haenlein GFW. Are camel milk proteins convenient to the nutrition of cow milk allergic children? Small Rumin Res. 2009;82:1–6.

26. Ehlayel MS, Hazeima KA, Al-Mesaifri F, Bener A. Camel milk: An alternative for cow's milk allergy in children. Allergy Asthma Proc. 2011;32:255–8.

27. Bøgh KL, Larsen JM. Chapter 19: Reducing allergenicity by proteolysis. Agents Chang Enzym Milk Dairy Prod. Springer: Berlin/Heidleberg, Germany; 2021. p. 499–524.

28. Bu G, Luo Y, Chen F, Liu K, Zhu T. Milk processing as a tool to reduce cow's milk allergenicity: A mini-review. Dairy Sci Technol. 2013;93:211–23.

29. Baby Formula [Internet]. [cited 2022 Apr 12]. Available from: https://camelicious.com/products/baby-formula-camel-milk

30. Smeekens JM, Kulis MD. Mouse Models of Food Allergy in the Pursuit of Novel Treatment

Modalities. Front Allergy. 2021;2:1–7.

31. Knippels LMJ, Penninks AH, Spanhaak S, Houben GF. Oral sensitization to food proteins : a Brown Norway rat model. 1998;28:368–75.

32. Dearman RJ, Kimber I. A mouse model for food allergy using intraperitoneal sensitization. Methods. 2007;41:91–8.

33. Brough HA, Nadeau KC, Sindher SB, Alkotob SS, Chan S, Bahnson HT, Leung DYM, Lack G. Epicutaneous sensitization in the development of food allergy: What is the evidence and how can this be prevented? Allergy Eur J Allergy Clin Immunol. 2020;75:2185–205.

34. Schülke S, Albrecht M. Mouse Models for Food Allergies: Where Do We Stand? Cells. 2019;8:546.

35. Adel-Patient K, Bernard H, Ah-Leung S, Créminon C, Wal JM. Peanut- and cow's milk-specific IgE, Th2 cells and local anaphylactic reaction are induced in Balb/c mice orally sensitized with cholera toxin. Allergy Eur J Allergy Clin Immunol. 2005;60:658–64.

36. Castan L, Bøgh KL, Maryniak NZ, Epstein MM, Kazemi S, O'Mahony L, Bodinier M, Smit JJ, van Bilsen JHM, Blanchard C, et al. Overview of in vivo and ex vivo endpoints in murine food allergy models: Suitable for evaluation of the sensitizing capacity of novel proteins? Allergy Eur J Allergy Clin Immunol. 2020;75:289–301.

37. Patrick JBE, Geoffrey BJ. Structure and Stability of Whey Proteins. Milk Proteins. Second Edi. Elsevier Inc.; 2014. p. 201–42. Available from: http://dx.doi.org/10.1016/B978-0-12-405171-3/00007-6

38. Snyder CS, Moodie DS. Hypoallergenic infant formulas. Pediatrics. 2000;106:346–9.

39. Halken S, Muraro A, de Silva D, Khaleva E, Angier E, Arasi S, Arshad H, Bahnson HT, Beyer K, Boyle R, et al. EAACI guideline: Preventing the development of food allergy in infants and young children (2020 update). Pediatr Allergy Immunol. 2021;32:843–58.

40. Martin CR, Ling PR, Blackburn GL. Review of infant feeding: Key features of breast milk and infant formula. Nutrients. 2016;8(5):1–11.

41. Panyam D, Kilara A. Enhancing the functionality of food proteins by enzymatic modification. Trends food Sci Technol. 1996;7:120–5.

42. Wróblewska B, Karamać M, Amarowicz R, Szymkiewicz A, Troszyńska A, Kubicka E. Immunoreactive properties of peptide fractions of cow whey milk proteins after enzymatic hydrolysis. Int J Food Sci Technol. 2004;39:839–50.

43. Bøgh KL, Madsen CB. Food Allergens: Is There a Correlation between Stability to Digestion and Allergenicity? Crit Rev Food Sci Nutr. 2016;56:1545–67.

44. Hochwallner H, Schulmeister U, Swoboda I, Spitzauer S, Valenta R. Cow's milk allergy: From allergens to new forms of diagnosis, therapy and prevention. Methods. 2014;66:22–33.

45. G.Bylund et al. Dairy Processing Handbook. Tetra Pak Processing System AB, SE-211 86,

Lund, Sweden; 2015.

46. Nowak-Wegrzyn A, Fiocchi A. Rare, medium, or well done? The effect of heating and food matrix on food protein allergenicity. Curr Opin Allergy Clin Immunol. 2009;9:234–7.

47. Rahaman T, Vasiljevic T, Ramchandran L. Effect of processing on conformational changes of food proteins related to allergenicity. Trends Food Sci Technol. 2016;49:24–34.

48. Villa C, Costa J, Oliveira MBPP, Mafra I. Bovine Milk Allergens: A Comprehensive Review. Compr Rev Food Sci Food Saf. 2018;17:137–64.

49. Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S, Herouet-Guicheney C, Holzhauser T, Shimojo R, van der Bolt N, et al. Food processing and allergenicity. Food Chem Toxicol. 2015;80:223–40.

50. Barbé F, Ménard O, Le Gouar Y, Buffière C, Famelart MH, Laroche B, Le Feunteun S, Dupont D, Rémond D. The heat treatment and the gelation are strong determinants of the kinetics of milk proteins digestion and of the peripheral availability of amino acids. Food Chem. 2013;136:1203–12.

51. Donato L, Guyomarc'h F. Formation and properties of the whey protein/κ-casein complexes in heated skim milk - A review. Dairy Sci Technol. 2009;89:3–29.

52. Gaspard SJ, Auty MAE, Kelly AL, O'Mahony JA, Brodkorb A. Isolation and characterisation of κ-casein/whey protein particles from heated milk protein concentrate and role of κ-casein in whey protein aggregation. Int Dairy J. 2017;73:98–108.

53. Felfoul I, Jardin J, Gaucheron F, Attia H, Ayadi MA. Proteomic profiling of camel and cow milk proteins under heat treatment. Food Chem. 2017;216:161–9.

54. Nielsen SD, Knudsen LJ, Bækgaard LT, Rauh V, Larsen LB. Influence of Lactose on the Maillard Reaction and Dehydroalanine-Mediated Protein Cross-Linking in Casein and Whey. Foods. 2022;11.

55. Nielsen SD, Le TT, Knudsen LJ, Rauh V, Poulsen NA, Larsen LB. Development and application of a multiple reaction monitoring mass spectrometry method for absolute quantification of lysinoalanine and lanthionine in dairy products. Int Dairy J. 2020;105.

56. van Lieshout GAA, Lambers TT, Bragt MCE, Hettinga KA. How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. Crit Rev Food Sci Nutr. 2020;60:2422–45.

57. Zibaee S, Hosseini SM al-reza, Yousefi M, Taghipour A, Kiani MA, Noras MR. Nutritional and Therapeutic Characteristics of Camel Milk in Children: A Systematic Review. Electron physician. 2015;7:1523–8.

58. Al haj OA, Al Kanhal HA. Compositional, technological and nutritional aspects of dromedary camel milk. Int Dairy J. 2010;20:811–21.

59. Swelum AA, El-Saadony MT, Abdo M, Ombarak RA, Hussein EOS, Suliman G, Alhimaidi AR,

Ammari AA, Ba-Awadh H, Taha AE, et al. Nutritional, antimicrobial and medicinal properties of Camel's milk: A review. Saudi J Biol Sci. 2021;28:3126–36.

60. Cho Y, Singh H, Creamer LK. Heat-induced interactions of β-lactoglobulin A and κ-casein B in a model system. J Dairy Res. 2003;70:61–71.

61. Fenaille F, Parisod V, Visani P, Populaire S, Tabet JC, Guy PA. Modifications of milk constituents during processing: A preliminary benchmarking study. Int Dairy J. 2006;16:728–39.

62. Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc. Nature Publishing Group; 2016;11:2301–19.

63. Hussein FA, Chay SY, Zarei M, Auwal SM, Hamid AA, Wan Ibadullah WZ, Saari N. Whey protein concentrate as a novel source of bifunctional peptides with angiotensin-i converting enzyme inhibitory and antioxidant properties: RSM study. Foods. 2020;9.

64. Doucet D, Otter DE, Gauthier SF, Foegeding EA. Enzyme-induced gelation of extensively hydrolyzed whey proteins by alcalase: Peptide identification and determination of enzyme specificity. J Agric Food Chem. 2003;51:6300–8.

65. Hedstrom L. Trypsin: a case study in the structural determinants of enzyme specificity. Biol Chem. 1996;377:465–70.

66. Bijl E, van Valenberg HJF, Huppertz T, van Hooijdonk ACM, Bovenhuis H. Phosphorylation of αS1-casein is regulated by different genes. J Dairy Sci. 2014;97:7240–6.

67. Li H, Zhao T, Li H, Yu J. Effect of Heat Treatment on the Property, Structure, and Aggregation of Skim Milk Proteins. Front Nutr. 2021;8:1–8.

68. Bennion BJ, Daggett V. The molecular basis for the chemical denaturation of proteins by urea. Proc Natl Acad Sci U S A. 2003;100:5142–7.

69. Alliegro MC. Effects of dithiothreitol on protein activity unrelated to thiol- disulfide exchange: For consideration in the analysis of protein function with cleland's reagent. Anal Biochem. 2000;282:102–6.

70. Skripak JM, Matsui EC, Mudd K, Wood RA. The natural history of IgE-mediated cow's milk allergy. J Allergy Clin Immunol. 2007;120:1172–7.

71. Zepeda-Ortega B, Goh A, Xepapadaki P, Sprikkelman A, Nicolaou N, Hernandez REH, Latiff AHA, Yat MT, Diab M, Hussaini B Al, et al. Strategies and Future Opportunities for the Prevention, Diagnosis, and Management of Cow Milk Allergy. Front Immunol. 2021;12:1–13.

72. Høst A, Halken S. A prospective study of cow milk allergy in Danish infants during the first 3 years of life: Clinical course in relation to clinical and immunological type of hypersensitivity reaction. Allergy. 1990;45:587–96.

73. M.M. Vonk, A.I. Kostadinova, M.V. Kopp BCAM van, Esch, L.E.M. Willemsen, L.M.J. Knippels JG. Dietary Interventions in Infancy. Allergy, Immun Toler Early Child. 2016. p. 261–84.

74. WHO. Guideline: Counselling of Women to Improve Breastfeeding Practices [Internet].

2018. Available from: https://www.who.int/publications/i/item/9789241550468

75. Fiocchi A, Dahda L, Dupont C, Campoy C, Fierro V, Nieto A. Cow's milk allergy: towards an update of DRACMA guidelines. World Allergy Organ J. 2016;9(35):1–11.

76. Vandenplas Y, Bhatia J, Shamir R, Agostoni C, Turck D, Staiano A, Szajewska H. Hydrolyzed formulas for allergy prevention. J Pediatr Gastroenterol Nutr. 2014;58:549–52.

77. American Academy of Pediatrics C of N. Hypoallergenic Infant Formulas. Pediatrics. 2000;106:346–9.

78. Vandenplas Y. Prevention and management of cow's milk allergy in non-exclusively breastfed infants. Nutrients. 2017;9:1–15.

79. Koletzko S, Niggemann B, Arato A, Dias JA, Heuschkel R, Husby S, Mearin ML, Papadopoulou A, Ruemmele FM, Staiano A, et al. Diagnostic approach and management of cow's-milk protein allergy in infants and children: Espghan gi committee practical guidelines. J Pediatr Gastroenterol Nutr. 2012;55:221–9.

80. Fleischer DM, Chan ES, Venter C, Spergel JM, Abrams EM, Stukus D, Groetch M, Shaker M, Greenhawt M. A Consensus Approach to the Primary Prevention of Food Allergy Through Nutrition: Guidance from the American Academy of Allergy, Asthma, and Immunology; American College of Allergy, Asthma, and Immunology; and the Canadian Society for Allergy and Clinical. J Allergy Clin Immunol Pract. 2021;9:22–43.

81. Joshi PA, Smith J, Vale S, Campbell DE. The Australasian Society of Clinical Immunology and Allergy infant feeding for allergy prevention guidelines. Med J Aust. 2019;210:89–93.

82. Von Berg A, Koletzko S, Grübl A, Filipiak-Pittroff B, Wichmann HE, Bauer CP, Reinhardt D, Berdel D. The effect of hydrolyzed cow's milk formula for allergy prevention in the first year of life: The German Infant Nutritional Intervention Study, a randomized double-blind trial. J Allergy Clin Immunol. 2003;111:533–40.

83. Lowe AJ, Hosking CS, Bennett CM, Allen KJ, Axelrad C, Carlin JB, Abramson MJ, Dharmage SC, Hill DJ. Effect of a partially hydrolyzed whey infant formula at weaning on risk of allergic disease in high-risk children: A randomized controlled trial. J Allergy Clin Immunol. 2011;128:360-365.e4.

84. Osborn DA, Sinn JKH, Jones LJ. Infant formulas containing hydrolysed protein for prevention of allergic disease. Cochrane Database Syst Rev. 2018;1–119.

85. Lowe AJ, Dharmage SC, Allen KJ, Tang ML, Hill DJ. The role of partially hydrolyzed whey formula for the prevention of allergic disease: Evidence and gaps. Expert Rev Clin Immunol. 2013;9:31–41.

86. Bøgh KL, Barkholt V, Madsen CB. The sensitising capacity of intact β-lactoglobulin is reduced by Co-administration with digested β-lactoglobulin. Int Arch Allergy Immunol. 2013;161:21–36.

87. Bøgh KL, Barkholt V, Madsen CB. Characterization of the Immunogenicity and Allergenicity of Two Cow's Milk Hydrolysates - A Study in Brown Norway Rats. Scand J Immunol.

2015;81:274–83.

88. Esch BCAM Van, Schouten B, Kivit S De, Hofman GA, Knippels MJ, Willemsen LEM, Garssen J. Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3 + regulatory T-cells in the mesenteric lymph nodes. 2011;22:820–6.

89. Liang X, Qian G, Sun J, Yang M, Shi X, Yang H, Wu J, Wang Z, Zheng Y, Yue X. Evaluation of antigenicity and nutritional properties of enzymatically hydrolyzed cow milk. Sci Rep. 2021;11:1–14.

90. Kiewiet MBG, van Esch BCAM, Garssen J, Faas MM, de Vos P. Partially hydrolyzed whey proteins prevent clinical symptoms in a cow's milk allergy mouse model and enhance regulatory T and B cell frequencies. Mol Nutr Food Res. 2017;61:1–12.

91. van Esch BCAM, Knipping K, Jeurink P, van der Heide S, Dubois AEJ, Willemsen LEM, Garssen J, Knippels LMJ. In vivo and in vitro evaluation of the residual allergenicity of partially hydrolysed infant formulas. Toxicol Lett. 2011;201:264–9.

92. Golkar A, Milani JM, Vasiljevic T. Altering allergenicity of cow's milk by food processing for applications in infant formula. Crit Rev Food Sci Nutr. 2019;59:159–72.

93. Bavaro SL, De Angelis E, Barni S, Pilolli R, Mori F, Novembre EM, Monaci L. Modulation of milk allergenicity by baking milk in foods: A proteomic investigation. Nutrients. 2019;11:1–15.

94. Uncuoglu A, Yologlu N, Simsek IE, Uyan ZS, Aydogan M. Tolerance to baked and fermented cow's milk in children with IgE-mediated and non-IgE-mediated cow's milk allergy in patients under two years of age. Allergol Immunopathol (Madr). 2017;45:560–6.

95. Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, Sampson HA. Tolerance to extensively heated milk in children with cow's milk allergy. J Allergy Clin Immunol. 2008;122.

96. Leonard SA, Nowak-Wegrzyn AH. Baked Milk and Egg Diets for Milk and Egg Allergy Management. Immunol Allergy Clin North Am. 2016;36:147–59.

97. Abbring S, Kusche D, Roos TC, Diks MAP, Hols G, Garssen J, Baars T, van Esch BCAM. Milk processing increases the allergenicity of cow's milk—Preclinical evidence supported by a human proof-of-concept provocation pilot. Clin Exp Allergy. 2019;49:1013–25.

98. Graversen KB, Ballegaard ASR, Kræmer LH, Hornslet SE, Sørensen L V., Christoffersen HF, Jacobsen LN, Untersmayr E, Smit JJ, Bøgh KL. Cow's milk allergy prevention and treatment by heat-treated whey—A study in Brown Norway rats. Clin Exp Allergy. 2020;50:708–21.

99. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J, Jensen-Jarolim E, Mayer L. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. Allergy Eur J Allergy Clin Immunol. 2008;63:882–90.

100. Verduci E, D'Elios S, Cerrato L, Comberiati P, Calvani M, Palazzo S, Martelli A, Landi M, Trikamjee T, Peroni DG. Cow's Milk Substitutes for Children: Nutritional Aspects of Milk from

Different Mammalian Species, Special Formula and Plant-Based Beverages. Nutrients. 2019;11:1739.

101. Tesse R, Paglialunga C, Braccio S, Armenio L. Adequacy and tolerance to ass's milk in an Italian cohort of children with cow's milk allergy. Ital J Pediatr. 2009;35:4–7.

102. Businco L, Giampietro PG, Lucenti P, Lucaroni F, Pini C, Di Felice G, Lacovacci P, Curadi C, Orlandi M. Allergenicity of mare's milk in children with cow's milk allergy. J Allergy Clin Immunol. 2000;105:1031–4.

103. Ehlayel M, Bener A, Abu Hazeima K, Al-Mesaifri F. Camel Milk Is a Safer Choice than Goat Milk for Feeding Children with Cow Milk Allergy. ISRN Allergy. 2011;2011:1–5.

104. Panel E, Nda A. Scientific Opinion on the suitability of goat milk protein as a source of protein in infant formulae and in follow-on formulae. EFSA J. 2012;10:1–18.

105. Rodríguez del Río P, Sánchez-García S, Escudero C, Pastor-Vargas C, Sánchez Hernández JJ, Pérez-Rangel I, Ibáñez MD. Allergy to goat's and sheep's milk in a population of cow's milk-allergic children treated with oral immunotherapy. Pediatr Allergy Immunol. 2012;23:128–32.

106. Polidori P, Vincenzetti S. Use of donkey milk in children with cow's milk protein allergy. Foods. 2013;2:151–9.

107. Fernandez A, Mills ENC, Koning F, Moreno FJ. Allergenicity Assessment of Novel Food Proteins: What Should Be Improved? Trends Biotechnol. 2021;39:4–8.

108. Muraro A, Werfel T, Hoffmann-Sommergruber K, Roberts G, Beyer K, Bindslev-Jensen C, Cardona V, Dubois A, Dutoit G, Eigenmann P, et al. EAACI Food Allergy and Anaphylaxis Guidelines: Diagnosis and management of food allergy. Allergy Eur J Allergy Clin Immunol. 2014;69:1008–25.

109. Prescott SL, Tang MLK. The Australasian Society of Clinical Immunology and Allergy position statement: Summary of allergy prevention in children. Med J Aust. 2005;182:464–7.

110. Berg A von, Filipiak-Pittroff B, Krämer U, Link E, Heinrich J, Koletzko S, Grübl A, Hoffmann U, Beckmann C, Reinhardt D, et al. The German Infant Nutritional Intervention Study (GINI) for the preventive effect of hydrolysed infant formulas in infants at high risk for allergic diseases. Design and selected results. Allergol Sel. 2017;1:28–39.

111. Vandenplas Y, Hauser B, Van den Borre C, Sacre L, Dab I. Effect of a whey hydrolysate prophylaxis of atopic disease. Ann Allergy. 1992;68:419–24.

112. Chandra RK. Five-year follow up of high-risk infants with family history of allergy exclusively breast-fed or fed partial whey hydrolysate, soy and conventional cow's milk formulas. Nutr Res. 1998;18:1395–411.

113. de Silva D, Halken S, Singh C, Muraro A, Angier E, Arasi S, Arshad H, Beyer K, Boyle R, du Toit G, et al. Preventing food allergy in infancy and childhood: Systematic review of randomised controlled trials. Pediatr Allergy Immunol. 2020;31:813–26.

114. Boyle RJ, Ierodiakonou D, Khan T, Chivinge J, Robinson Z, Geoghegan N, Jarrold K, Afxentiou T, Reeves T, Cunha S, et al. Hydrolysed formula and risk of allergic or autoimmune disease: Systematic review and meta-analysis. BMJ. 2016;352.

115. Iwamoto H, Matsubara T, Okamoto T, Matsumoto T, Yoshikawa M, Takeda Y. Ingestion of casein hydrolysate induces oral tolerance and suppresses subsequent epicutaneous sensitization and development of anaphylaxis reaction to casein in mice. Int Arch Allergy Immunol. 2019;179:221–30.

116. Graversen KB, Larsen JM, Pedersen SS, Sørensen LV, Christoffersen HF, Jacobsen LN, Halken S, Licht TR, Bahl MI, Bøgh KL. Partially Hydrolysed Whey Has Superior Allergy Preventive Capacity Compared to Intact Whey Regardless of Amoxicillin Administration in Brown Norway Rats. Front Immunol. 2021;12:1–16.

117. Fritsche R. Animal models in food allergy: assessment of allergenicity and preventive activity of infant formulas. Toxicol Lett. 2003;141:303–9.

118. Jensen LH, Larsen JM, Madsen CB, Laursen RR, Jacobsen LN, Bøgh KL. Preclinical Brown Norway Rat Models for the Assessment of Infant Formulas in the Prevention and Treatment of Cow's Milk Allergy. Int Arch Allergy Immunol. 2019;178:307–14.

119. Chikhi A, Elmecherfi KE, Bernard H, Cortes-Perez N, Kheroua O, Saidi D, Adel-Patient K. Evaluation of the efficiency of hydrolyzed whey formula to prevent cow's milk allergy in the BALB/c mouse model. Pediatr Allergy Immunol. 2019;30:370–7.

120. Vita D, Passalacqua G, Di Pasquale G, Caminiti L, Crisafulli G, Rulli I, Pajno GB. Ass's milk in children with atopic dermatitis and cow's milk allergy: Crossover comparison with goat's milk. Pediatr Allergy Immunol. 2007;18:594–8.

121. Docena G, Rozenfeld P, Fernández R, Fossati CA. Evaluation of the residual antigenicity and allergenicity of cow's milk substitutes by in vitro tests. Allergy Eur J Allergy Clin Immunol. 2002;57:83–91.

122. Järvinen KM, Chatchatee P. Mammalian milk allergy: Clinical suspicion, cross-reactivities and diagnosis. Curr Opin Allergy Clin Immunol. 2009;9:251–8.

123. Mati A, Senoussi-Ghezali C, Si Ahmed Zennia S, Almi-Sebbane D, El-Hatmi H, Girardet JM. Dromedary camel milk proteins, a source of peptides having biological activities – A review. Int Dairy J. 2017;73:25–37.

124. Aburiziza AJ. Cross reactivity between cow's and camel's milk in the infant population of Saudi Arabia. Curr Pediatr Res. 2020;24:175–9.

125. Restani P, Gaiaschi A, Plebani A, Beretta B, Cagavni G, Fiocchi A, Poiesi C, Velona T, Ugazio AG, Galli CL. Cross-reactivity between milk proteins from different animal species. Clin Exp Allergy. 1999;29:997–1004.

126. Graversen KB, Bahl MI, Larsen JM, Ballegaard ASR, Licht TR, Bøgh KL. Short-Term Amoxicillin-Induced Perturbation of the Gut Microbiota Promotes Acute Intestinal Immune

Regulation in Brown Norway Rats. Front Microbiol. 2020;11:1–14.

127. Locke AV, Larsen JM, Graversen KB, Licht TR, Bahl MI, Bøgh KL. Amoxicillin does not affect the development of cow's milk allergy in a Brown Norway rat model. Scand J Immunol. 2022;1–13.

128. Thang CL, Zhao X. Effects of orally administered immunodominant T-cell epitope peptides on cow ' s milk protein allergy in a mouse model. FRIN. Elsevier Ltd; 2015;71:126–31. Available from: http://dx.doi.org/10.1016/j.foodres.2015.03.011

129. Hospital ST, Children K. Oral Immunotherapy Using Partially Hydrolyzed Formula for Cow ' s Milk Protein Allergy : A Randomized , Controlled Trial. 2018;8555:259–68.

130. van Esch BCAM, Schouten B, de Kivit S, Hofman GA, Knippels LMJ, Willemsen LEM, Garssen J. Oral tolerance induction by partially hydrolyzed whey protein in mice is associated with enhanced numbers of Foxp3 + regulatory T-cells in the mesenteric lymph nodes. Pediatr Allergy Immunol. 2011;22:820–6.

131. Ciprandi G. Clinical bystander effect exerted by allergen immunotherapy: A hypothesis. Eur Ann Allergy Clin Immunol. 2015;47:62–3.

132. Thang CL, Zhao X. Effects of orally administered immunodominant T-cell epitope peptides on cow's milk protein allergy in a mouse model. Food Res Int. 2015;71:126–31.

133. Circassia Announces Top-Line Results from Cat Allergy Phase III Study [Internet]. [cited 2022 May 20]. Available from: https://www.circassia.com/media/press-releases/circassia-announces-top-line-results-from-cat-allergy-phase-iii-study/

134. Rupa P, Mine Y. Oral immunotherapy with immunodominant T-cell epitope peptides alleviates allergic reactions in a Balb/c mouse model of egg allergy. Allergy Eur J Allergy Clin Immunol. 2012;67:74–82.

135. Yang M, Yang C, Mine Y. Multiple T cell epitope peptides suppress allergic responses in an egg allergy mouse model by the elicitation of forkhead box transcription factor 3- and transforming growth factor-β-associated mechanisms. Clin Exp Allergy. 2010;40:668–78.

136. Motosue MS, Dominquez T, Sciancalepore A, Pineda D, Mehrotra A, Hoyte L, Nadeau K. Bystander Suppression in Food Allergy. J Allergy Clin Immunol. 2013. p. 335.

137. van der Valk J, Nagl B, van Wljk RG, Bohle B, de Jong N. nutrients The E ff ect of Birch Pollen Immunotherapy on Apple and rMal d 1 Challenges in Adults with Apple Allergy. 2020;1:1–11.

138. Hansen KS, Khinchi MS, Skov PS, Bindslev-Jensen C, Poulsen LK, Malling HJ. Food allergy to apple and specific immunotherapy with birch pollen. Mol Nutr Food Res. 2004;48:441–8.

139. Mauro M, Russello M, Incorvaia C, Gazzola G, Frati F, Moingeon P, Passalacqua G. Birch-apple syndrome treated with birch pollen immunotherapy. Int Arch Allergy Immunol. 2011;156:416–22.

140. Haka J, Niemi MH, Iljin K, Reddy VS, Takkinen K, Laukkanen M. Isolation of Mal d 1 and Api g 1 - specific recombinant antibodies from mouse IgG Fab fragment libraries – Mal d 1-specific antibody exhibits cross-reactivity against Bet v 1. BMC Biotechnol. 2015;1–15.

141. Elizur A, Appel MY, Nachshon L, Levy MB, Rigbi NE-, Koren Y, Holmqvist M, Porsch H, Lidholm J, Goldberg MR. Cashew oral immunotherapy for desensitizing cashew- - pistachio allergy (NUT CRACKER study). 2022;1–10.

142. Willison LN, P. Tawde , J. M. Robotham , R. M. Penney IV , S. S. Teuberw SKS and KHR. Pistachio vicilin , Pis v 3 , is immunoglobulin E-reactive and cross-reacts with the homologous cashew allergen , Ana o 1 Clinical and Experimental Allergy. 2008;1:1229–38.

143. Pomés A, Davies JM, Gadermaier G, Hilger C, Holzhauser T, Lidholm J, Lopata AL, Mueller GA, Nandy A, Radauer C, et al. WHO/IUIS Allergen Nomenclature: Providing a common language. Mol Immunol. 2018;100:3–13.

144. Sélo I, Clément G, Bernard H, Chatel JM, Créminon C, Peltre G, Wal JM. Allergy to bovine β-lactoglobulin: Specificity of human IgE to tryptic peptides. Clin Exp Allergy. 1999;29:1055–63.

145. Pena RN, Folch JM, Sánchez A, Whitelaw CBA. Chromatin structures of goat and sheep β-lactoglobulin gene differ. Biochem Biophys Res Commun. 1998;252:649–53.

146. Behrouz S, Saadat S, Memarzia A, Sarir H, Folkerts G, Boskabady MH. The Antioxidant, Anti-Inflammatory and Immunomodulatory Effects of Camel Milk. Front Immunol. 2022;13:1–17.

147. Schoemaker AA, Sprikkelman AB, Grimshaw KE, Roberts G, Grabenhenrich L, Rosenfeld L, Siegert S, Dubakiene R, Rudzeviciene O, Reche M, et al. Incidence and natural history of challenge-proven cow's milk allergy in European children - EuroPrevall birth cohort. Allergy Eur J Allergy Clin Immunol. 2015;70:963–72.

148. WHO. Infant and young child nutrition: Global strategy on infant and young child feeding. Proc Fifty Fifth World Heal Assem. 2002;53:1–18.

149. Ludman S, Shah N, Fox AT. Managing cows' milk allergy in children. BMJ. 2013;347:1–8.

150. Host A, Halken S. Hypoallergenic formulas - when, to whom and how long: after more than 15 years we know the right indication! Allergy. 2004;59:45–52.

151. Meyer R, Groetch M, Venter C. When Should Infants with Cow's Milk Protein Allergy Use an Amino Acid Formula? A Practical Guide. J Allergy Clin Immunol Pract. 2018;6:383–99.

152. Shabo Y, Barzel R, Margoulis M, Yagil R. Camel milk for food allergies in children. Isr Med Assoc J. 2005;7:796–8.

153. Ehlayel M, Bener A, Abu Hazeima K, Al-Mesaifri F. Camel Milk Is a Safer Choice than Goat Milk for Feeding Children with Cow Milk Allergy. ISRN Allergy. 2011;2011:1–5.

154. Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, Sampson HA. Tolerance to extensively heated milk in children with cow's milk allergy. J Allergy Clin Immunol.

2008;122.

155. Kim JS, Nowak-Wgrzyn A, Sicherer SH, Noone S, Moshier EL, Sampson HA. Dietary baked milk accelerates the resolution of cow's milk allergy in children. J Allergy Clin Immunol. 2011;128:125-131.e2.

156. Esmaeilzadeh H, Alyasin S, Haghighat M, Nabavizadeh H, Esmaeilzadeh E, Mosavat F. The effect of baked milk on accelerating unheated cow's milk tolerance: A control randomized clinical trial. Pediatr Allergy Immunol. 2018;29:747–53.

157. Broekman HCH, Eiwegger T, Upton J, Bøgh KL. IgE - the main player of food allergy. Drug Discov Today Dis Model. 2015;17–18:37–44.

158. Nowak-Wegrzyn AH, Lawson K, Masilamani M, Kattan J, Bahnson HT, Sampson HA. Increased tolerance to less extensively heat-denatured (baked) milk products in milk-allergic children. J Allergy Clin Immunol Pract. 2018;6:486–95.

159. Bu G, Luo Y, Zheng Z, Zheng H. Effect of heat treatment on the antigenicity of bovine α-lactalbumin and β-lactoglobulin in whey protein isolate. Food Agric Immunol. 2009;20:195–206.

160. Ciepiela O, Zwiazek J, Zawadzka-Krajewska A, Kotula I, Kulus M, Demkow U. Basophil activation test based on the expression of CD203C in the diagnostic of cow milk allergy in children. Eur J Med Res. 2010;15:21–6.

161. Ford LS, Bloom KA, Nowak-Węgrzyn AH, Shreffler WG, Masilamani M, Sampson HA. Basophil reactivity, wheal size, and immunoglobulin levels distinguish degrees of cow's milk tolerance. J Allergy Clin Immunol. 2013;131.

162. Morisawa Y, Kitamura A, Ujihara T, Zushi N, Kuzume K, Shimanouchi Y, Tamura S, Wakiguchi H, Saito H, Matsumoto K. Effect of heat treatment and enzymatic digestion on the B cell epitopes of cow's milk proteins. Clin Exp Allergy. 2009;39:918–25.

163. Ehlayel M, Bener A. Camel's milk allergy. Allergy Asthma Proc. 2018;39:384–8.

164. Al-Hammadi S, El-Hassan T, Al-Reyami L. Anaphylaxis to camel milk in an atopic child. Allergy. 2010;65:1622–3.

165. Micinskia J, KowaIskil IM, Zwierzchowskia G, Szarelt J, Pieroyñski B, Zablocka E. Characteristics of cow's milk proteins including allergenic properties and methods for its reduction. Polish Ann Med. 2013;20:69–76.

166. Levy M, Nachshon L, Goldberg M, Elizur A, Katz Y. Cross-desensitization to goat and sheep milk following cow's milk oral immunotherapy. J Allergy Clin Immunol Pract. 2014;133.

167. Floris R, Lambers T, Alting A, Kiers J. Trends in infant formulas: A dairy perspective. Improv Saf Qual Milk Improv Qual Milk Prod. Woodhead Publishing Limited; 2010. p. 454–74.

168. Farah Z, Rettenmaier R, Attkins D. Vitamin content of camel milk. Int J Vitam Nutr Res. 1992;62:30–3.

169. Vincenzetti S, Cammertoni N, Rapaccetti R, Santini G, Klimanova Y, Zhang J-J, Polidori P.

Nutraceutical and Functional Properties of Camelids’ Milk. Beverages. 2022;8:12.

Supplementary material

Supplementary material for Manuscript III

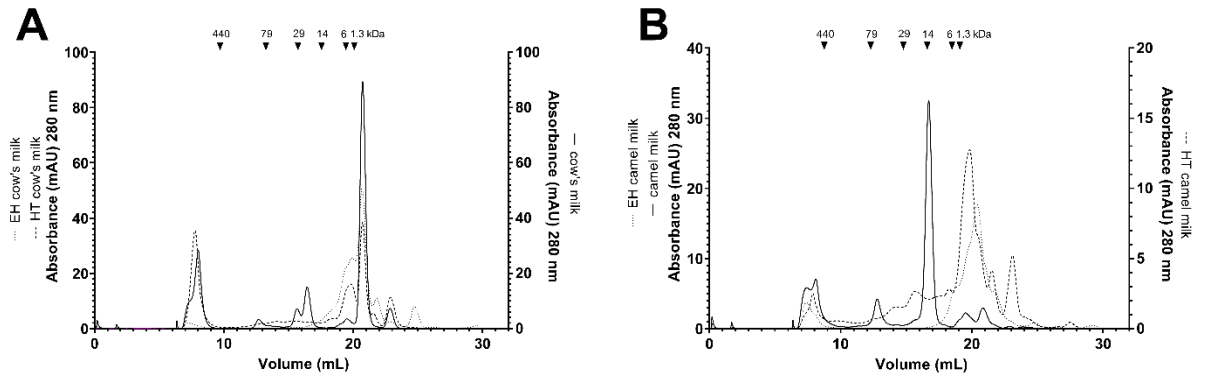

Figure S1. Gel permeation chromatography of milk products under physiological conditions at 280 nm. (A) Protein separation profile of cow's milk, --- HT cow's milk and ···· EH cow's milk. **(B)** Protein separation profile of camel milk, --- HT camel milk and ···· EH camel milk.

Table S1. Accession numbers (https://www.uniprot.org) of proteins from cow's and camel milk used in the analysis of raw data from LC-MS/MS analysis. NA, not identified.

Protein	Cow's milk - accession numbers	Camel milk - accession numbers
β-casein	P02666	Q9TVD0
αs1-casein	P02662	O97943
αs2-casein	O97944	O97944
κ-casein	P02668	L0P3Z7
β-lactoglobulin	P02754	NA
α-lactalbumin	P00711	P00710
Serum albumin	P02769	XP_010981066.1
Glycosylation-dependent cell adhesion molecule-1	P80195	P15522
Whey acidic protein	NA	P09837
Lactoferrin	P24627	AHJ37525.1

Supplementary material for Manuscript V

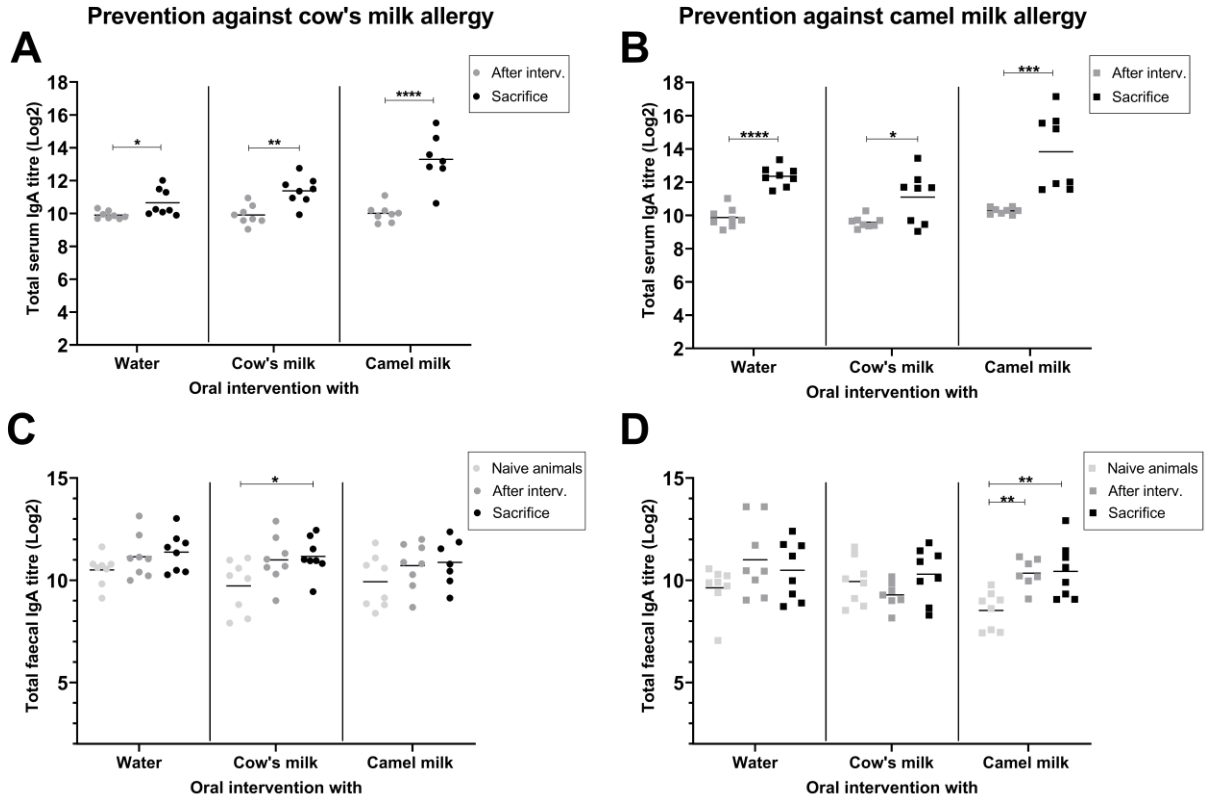

Figure S1. (A) Total IgA in serum from rats intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(B)** Total IgA in serum from rats intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy. **(C)** Total IgA in faeces from rats intervened with either water, cow's milk or camel milk and tested for prevention against cow's milk allergy. **(D)** Total IgA in faeces from rats intervened with either water, cow's milk or camel milk and tested for prevention against camel milk allergy**.** Each color represents different time points of the experiment: (●),(●) beginning of experiment (naïve rats), (●),(■) after intervention phase (after interv.) and (●),(■) the day of sacrifice. Either a parametric t-test **(A,B)** or an one-way ANOVA followed by Bonferroni post-test **(C,D)** were applied. Statistically significant differences are shown as *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001 and ****P≤ 0.0001.

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