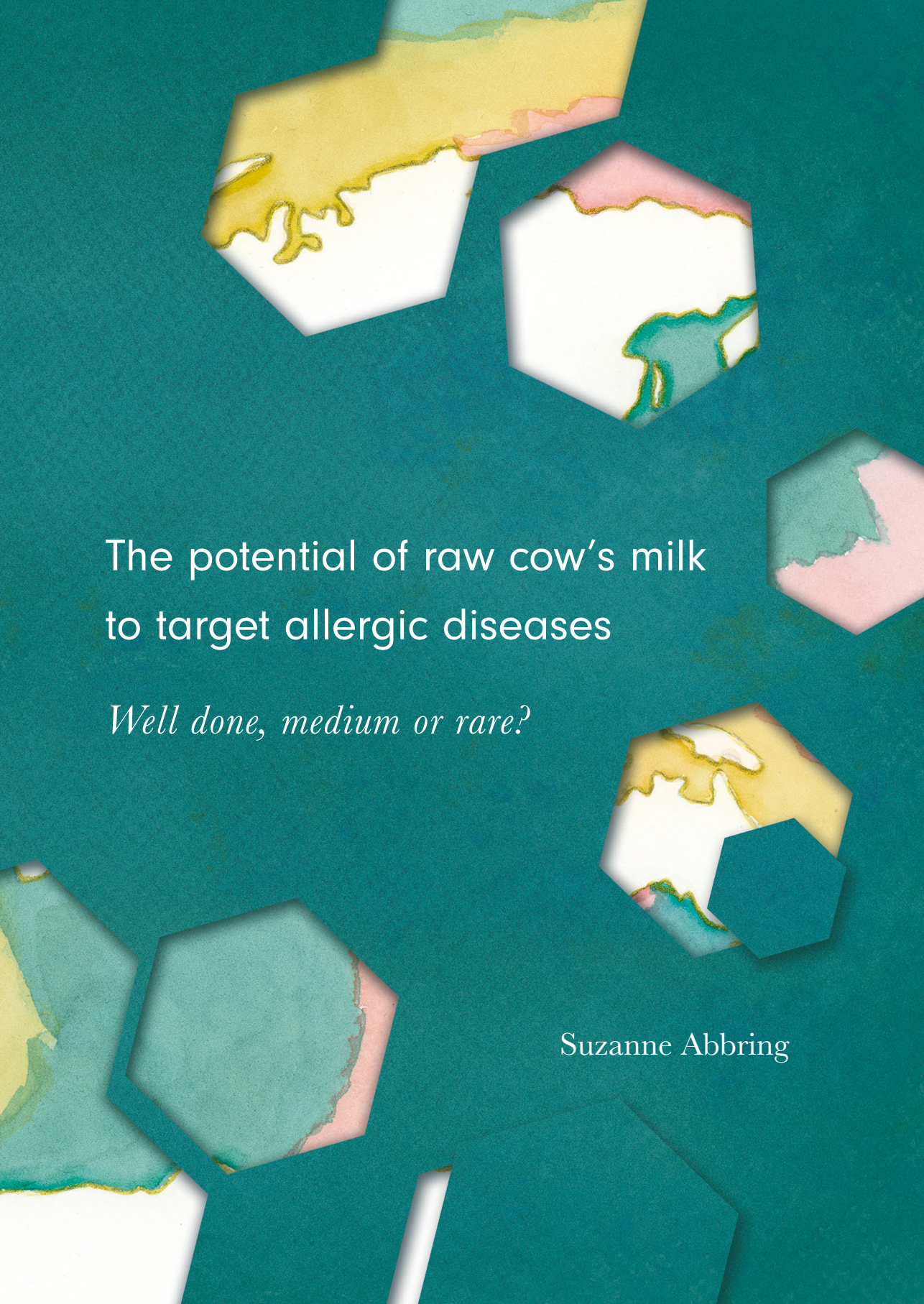

The potential of raw cow's milk to target allergic diseases

Well done, medium or rare?

Suzanne Abbring

THE POTENTIAL OF RAW COW'S MILK TO TARGET ALLERGIC DISEASES

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THE POTENTIAL OF RAW COW'S MILK TO TARGET ALLERGIC DISEASES

'WELL DONE, MEDIUM OR RARE?'

De potentie van rauwe koemelk om allergieën tegen te gaan
(met een samenvatting in het Nederlands)

Proefschrift

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door

Suzanne Abbring
geboren op 12 oktober 1989 te Hoorn

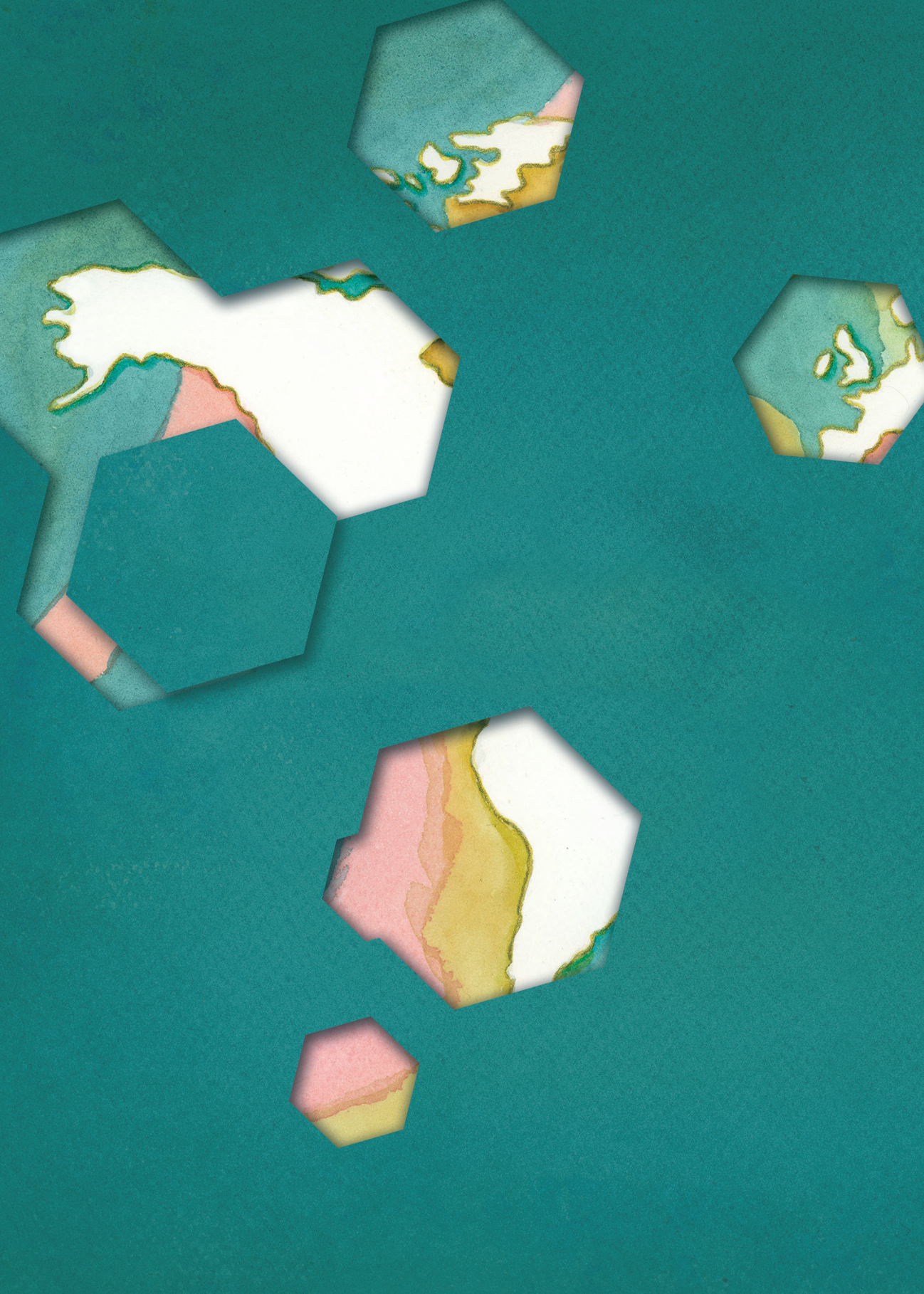
Promotor: Prof. dr. J. Garssen

Copromotor: Dr. B.C.A.M. van Esch

‘A lack of passion can do more harm than simple inexperience’

– Marcel Minnaert –

CONTENTS



CHAPTER 1

General introduction

Raw, unprocessed, cow's milk consumption is discussed with passion. Proponents argue that raw milk has numerous health benefits, while opponents advise against its consumption because of the risk of contamination by pathogens and the lack of scientifically substantiated evidence. The potential presence of pathogens is reason for governmental agencies to prohibit or strongly discourage the consumption of raw cow's milk. At the same time, the prevailing trend towards more natural food products seems to increase the preference for its consumption.

HISTORY OF COW'S MILK CONSUMPTION

Thousands of years ago, humans started to consume cow's milk as a readily available source of protein and energy (1). At that time, cow's milk was safely consumed raw, but as society industrialized around the 19th century, increased milk production and distribution led to outbreaks of milk-borne diseases. Contamination of milk with pathogenic bacteria like *Mycobacterium tuberculosis*, *Listeria*, *Salmonella*, *Campylobacter*, Enterohemorrhagic *Escherichia coli*, and Shigatoxigenic *Escherichia coli* appeared to be the underlying cause of morbidity and mortality associated with raw milk consumption (2-4). Invented by Louis Pasteur in 1864 to improve the shelf life of wine and first applied to milk by professor N.J. Fjord in 1870, pasteurization was subsequently introduced to solve the critical issue of milk-borne infections (2, 3).

Pasteurization of commercial milk became compulsory in the late 19th century and since then several processing steps have been implemented (5). After pasteurization (71-74 °C for 15-40 s), various other heat treatments such as sterilization (110-120 °C for 10-20 min) and ultra-high temperature (UHT) processing (135-145 °C for 0.5-4 s) were introduced to prolong shelf life (6, 7). In addition, milk is nowadays often standardized to obtain the desired fat content and homogenized to prevent the separation of a cream layer (8). Other steps in the processing chain are machine milking, cooling, cold storage, and packaging (9). Each of these processing steps preserves milk along the supply chain, which makes it easy for today's Western society to consume milk in everyday life.

Milk processing not only ensures microbiological safety, it also induces changes in the milk composition. Especially the fat content and the heat-sensitive milk components are affected by industrially applied processing steps. Standardization, for example, reduces the fat content of the milk from the natural minimum of 3.25% (8). Homogenization disrupts the fat globules by pumping milk under high pressure through narrow pipes and heat treatment structurally alters heat-sensitive milk components (9, 10). These profound effects on the milk structure do not affect the nutritional value of the milk but they might bear other disadvantages.

RAW COW'S MILK

Despite the potential risk of life-threatening infections, raw cow's milk consumption is still rather common among dairy farming families and to a certain extent also among rural non-farming families. These families made it possible to investigate potential beneficial health effects related to raw cow's milk consumption (11).

In the last decades of the 20th century, the prevalence of allergic diseases markedly increased (12). This increase is mainly evident in affluent Western countries and is therefore often explained by the loss of rural living conditions and associated changes in diet and lifestyle, resulting in a decreased microbial exposure in early life (13). This is also referred to as the 'hygiene hypothesis', and in accordance with this hypothesis it has been shown that growing up on a farm confers protection against allergic diseases (14-18). This protective 'farm effect' was demonstrated in many populations and sustained into adult life (19).

The consumption of raw cow's milk has been identified as one of the farm-associated exposures contributing to this protective effect. Numerous large-scale epidemiological studies among European populations have repeatedly shown that the consumption of raw cow's milk early in life is associated with a reduced risk of developing asthma and allergies (20-25). This allergy-protective effect was found to be independent of farm-related co-exposures and farming status, suggesting that a general population might equally benefit from the consumption of raw cow's milk (20-22).

IMPACT OF MILK PROCESSING

Interestingly, milk processing seems to destroy the capacity of raw cow's milk to protect against allergic diseases. In contrast to raw milk, industrially processed milk was not able to reduce allergy risk (24, 25). As described above, milk processing significantly changes the composition of the milk, so that processed cow's milk differs from raw cow's milk in many respects. These changes might explain why the beneficial raw milk effect appears to be destroyed upon milk processing. Heat treatment, for instance, not only destroys pathogens, but also beneficial bacteria which could act as probiotics. Heating furthermore structurally alters heat-sensitive components, such as proteins, which might consequently lose functionality (11, 26). Homogenization causes a reduction of fat globule size and a concurrent increase in the milk fat surface area. To cover this increase, milk proteins, particularly caseins and β-lactoglobulin, are included (27). Since these are the main milk allergens, homogenization might alter allergen presentation to the immune system (9).

Since milk processing predominantly affects the fat content of the milk and the heat-sensitive milk components, these constituents are likely to contribute to the observed protection. Part of the asthma-protective effect of raw cow's milk was indeed explained by a higher fat content and particularly, higher *n*-3 polyunsaturated fatty acids levels compared to industrially processed milk (25). In addition, frequent consumption of milk fat-containing products such as full cream milk and butter has been associated with a reduced risk of asthma symptoms (28). Similarly, the heat-sensitive whey protein fraction of raw milk was found to be inversely related to asthma risk (24). Many of the proteins present in this whey fraction, such as immunoglobulins, lactoferrin, alkaline phosphatase, and anti-inflammatory cytokines, have immunomodulatory capacities and heating them can lead to loss of functionality (29).

Besides *n*-3 polyunsaturated fatty acids and whey proteins, there are several other raw milk constituents with immunomodulatory functionalities that might explain how raw cow's milk consumption can reduce the risk of allergic diseases. The microbial content of the milk, oligosaccharides, antioxidants, vitamins, and exosomal microRNAs are all hypothesized to contribute to the protective effects (11, 30, 31). It is speculated that the many bioactive components present in raw cow's milk act together to create a regulatory environment which favors unresponsiveness upon allergen exposure. During milk processing, some of these immunomodulatory components are removed and/or inactivated, resulting in a loss of the allergy-protective effect.

Despite the promising results of the many epidemiological studies conducted, the observed associations do not confirm a causal relationship. Without proof of causality, the perceived health benefits will never outweigh the potential risks and the debate around raw cow's milk consumption will keep on going. Controlled intervention studies to show causality have not been conducted due to safety and ethical reasons, but preclinical models might offer a solution. Even though the risks of raw cow's milk consumption are rather low when produced under strict hygienic and microbiological standards, a zero-risk can never be attained. Besides confirming a cause-effect relationship, scientific efforts should therefore focus on deciphering the raw milk components involved and the underlying mechanisms. This knowledge is crucial to develop alternatives to raw cow's milk consumption such as mildly processed milk, in which bioactive raw milk components are retained, or the addition of specific raw milk components to heat-treated milk. Since there are currently no effective preventive approaches for allergic diseases, a potential natural solution, as offered by the consumption of raw cow's milk or the use of its native ingredients, must be thoroughly investigated before being labeled as nonsense.

AIM AND OUTLINE OF THIS THESIS

This thesis aims to investigate whether the currently described associations between raw cow's milk consumption and the prevention of allergic diseases can be confirmed in *in vivo* mouse models to demonstrate causality. Since the consumption of raw cow's milk is prohibited or strongly discouraged by governmental agencies due to the potential presence of pathogens, the raw milk components involved in the allergy-protective effects and the underlying mechanisms were also investigated to support the development of alternatives in the future.

Chapter 2 describes the scientific background behind the studies performed in this thesis. Current literature regarding the role of raw cow's milk in allergic diseases is reviewed. Since mainly heat treatment seems to destroy the allergy-protective effects of raw cow's milk, focus was on the heat-sensitive whey proteins. The immunological effects of several of these whey proteins, their potential contribution to the allergy-protective effects of raw cow's milk and the consequences of heat treatment were discussed.

Most of the epidemiological studies showing a protective effect of raw cow's milk consumption are focused on asthma. In **Chapter 3**, we therefore used a well-defined murine house dust mite-induced allergic asthma model to investigate whether the observed associations could be strengthened by causality. In accordance with the existing epidemiological studies, raw cow's milk prevented the development of allergic asthma. Strong protective effects on airway hypersensitivity and airway inflammation were observed and the suppression of local type 2 cytokine levels seemed to be crucial. Heat-treated raw milk did not show an asthma-protective effect, indicating the involvement of heat-sensitive raw milk components.

Chapter 4 demonstrates that raw cow's milk is also protective in a murine model for food allergy. Raw milk exposure for eight days, prior to sensitization and challenge with ovalbumin (OVA), suppressed allergic symptoms, indicating that raw milk is able to induce tolerance to an unrelated, non-milk, food allergen. Treatment with processed shop milk did not confer protection against OVA-induced allergic symptoms. Since epigenetic regulation is an important mechanism by which environmental factors interact with genes involved in asthma and allergy development, histone acetylation of T cell genes was also assessed in this chapter. We showed that raw milk is able to modulate gene expression through epigenetic mechanisms and concluded that raw milk might have induced tolerance by targeting histone marks on T cell-related genes.

Since raw milk and commercially available processed milk differ in many respects, **Chapter 5** focusses on milk from the same milk source differing in only one processing step, to gain more insight in the raw milk components involved in the allergy-protective effect and the underlying mechanisms. The suppression of food allergic symptoms by raw milk was shown to be retained after skimming but abolished after pasteurization of the milk, indicating that not the fat content but the heat-sensitive milk components are underlying the allergy-protective effects of raw cow's milk. The protection by raw and skimmed raw cow's milk was accompanied by an induction of tolerance-associated cell types in the mesenteric lymph nodes. In addition, this chapter shows that adding alkaline phosphatase to heat-treated milk might be an interesting alternative to raw cow's milk consumption, as spiking pasteurized milk with alkaline phosphatase restored the protective effects.

The heat-sensitive whey protein fraction of raw milk is a likely source of the allergy-protective components. In **Chapter 6**, we therefore aimed to achieve a better understanding of the mechanistic relation between heat damage to whey proteins and allergy development. We used proteomics to compare the native whey protein profile of raw cow's milk and milk heated at various temperatures (50-80 °C). Changes in the native protein profile were subsequently related to the capacity of the milk to prevent the development of OVA-induced food allergy. A substantial loss of native whey proteins was observed from 75 °C, but immunologically active whey proteins already started to denature from 65 °C. Interestingly, the loss of immunologically active whey proteins coincided with the temperature at which a loss of allergy protection was observed in the murine food allergy model. The results thereby demonstrate that immunologically active whey proteins that denature around 65 °C are of importance for the allergy-protective capacity of raw cow's milk.

Chapter 7 zooms in on four whey proteins with promising immunomodulatory functionalities. Commercially available shop milk was spiked with lactoferrin, IgG, alkaline phosphatase, osteopontin, or the combination of the four, to assess the ability of these whey proteins to restore the allergy-protective effect lost after milk processing. In a murine OVA-induced food allergy model, all four components showed protective effects and we therefore concluded that supplementing heat-treated milk with bioactive whey proteins, as an alternative to raw cow's milk, is a promising preventive approach for allergic diseases.

The previous chapters all focused on the asthma- and food (i.e. OVA) allergy-protective capacity of raw cow's milk consumption and showed an adverse effect of milk processing. Whether milk processing also affects the capacity of the milk to induce a milk allergic response, i.e. the allergenicity of the milk, is investigated in **Chapter 8**. In this chapter, we demonstrated that raw cow's milk and native whey proteins have a lower allergenicity than

their processed counterparts. These findings were extensively shown in a murine cow's milk allergy model and were confirmed in a proof-of-concept provocation trial with milk allergic children. This chapter thereby provides evidence that milk processing negatively influences the allergenicity of milk.

Chapter 9 describes a direct inhibitory effect of raw cow's milk on the allergic effector response *in vitro*. Next to the earlier described capacity to modulate T cell responses, this chapter shows that raw cow's milk is also able to influence mast cell activation. Mast cell activation was not affected by heated raw milk and shop milk, which once again demonstrates a loss of allergy protection after milk processing and more specifically after heat treatment. Raw milk fractionation based on size showed that the heat-sensitive raw milk components responsible for the reduced mast cell activation are likely to have a molecular weight of > 37 kDa.

Finally, the findings of this thesis are summarized and discussed in **Chapter 10** in order to provide an answer to the question 'well done, medium or rare?'. A graphic outline of this thesis is depicted in **Figure 1**.

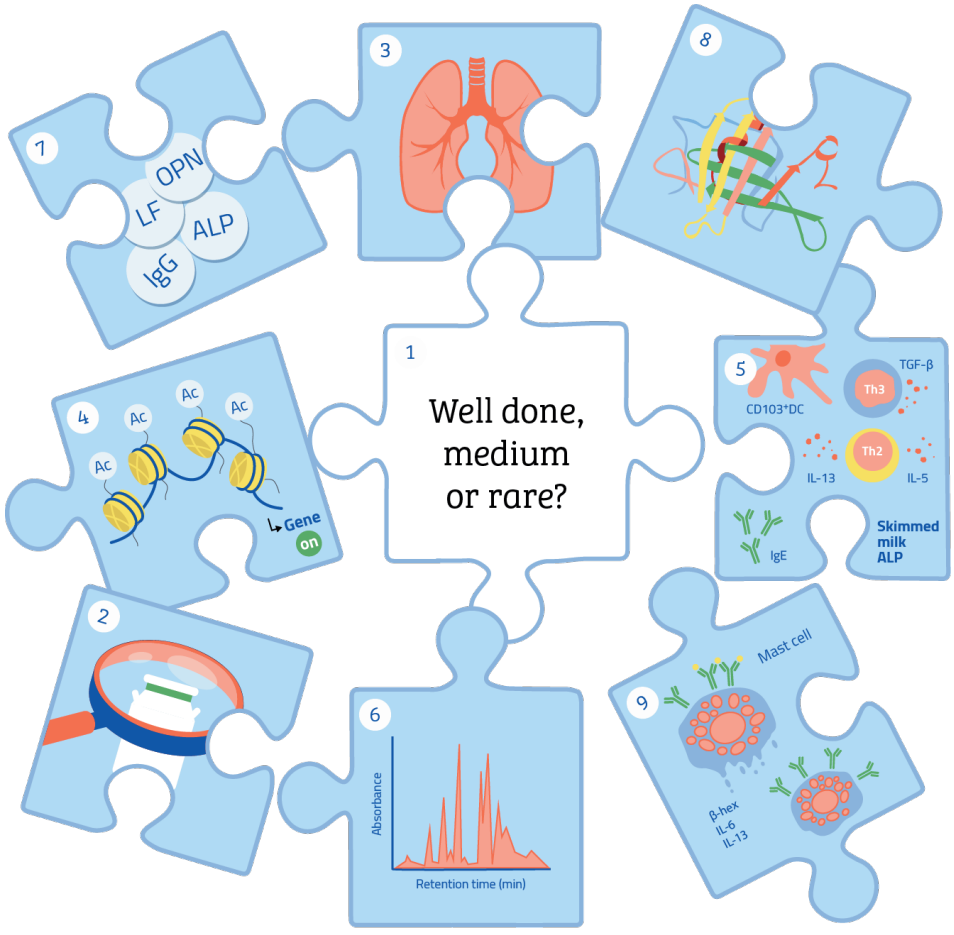
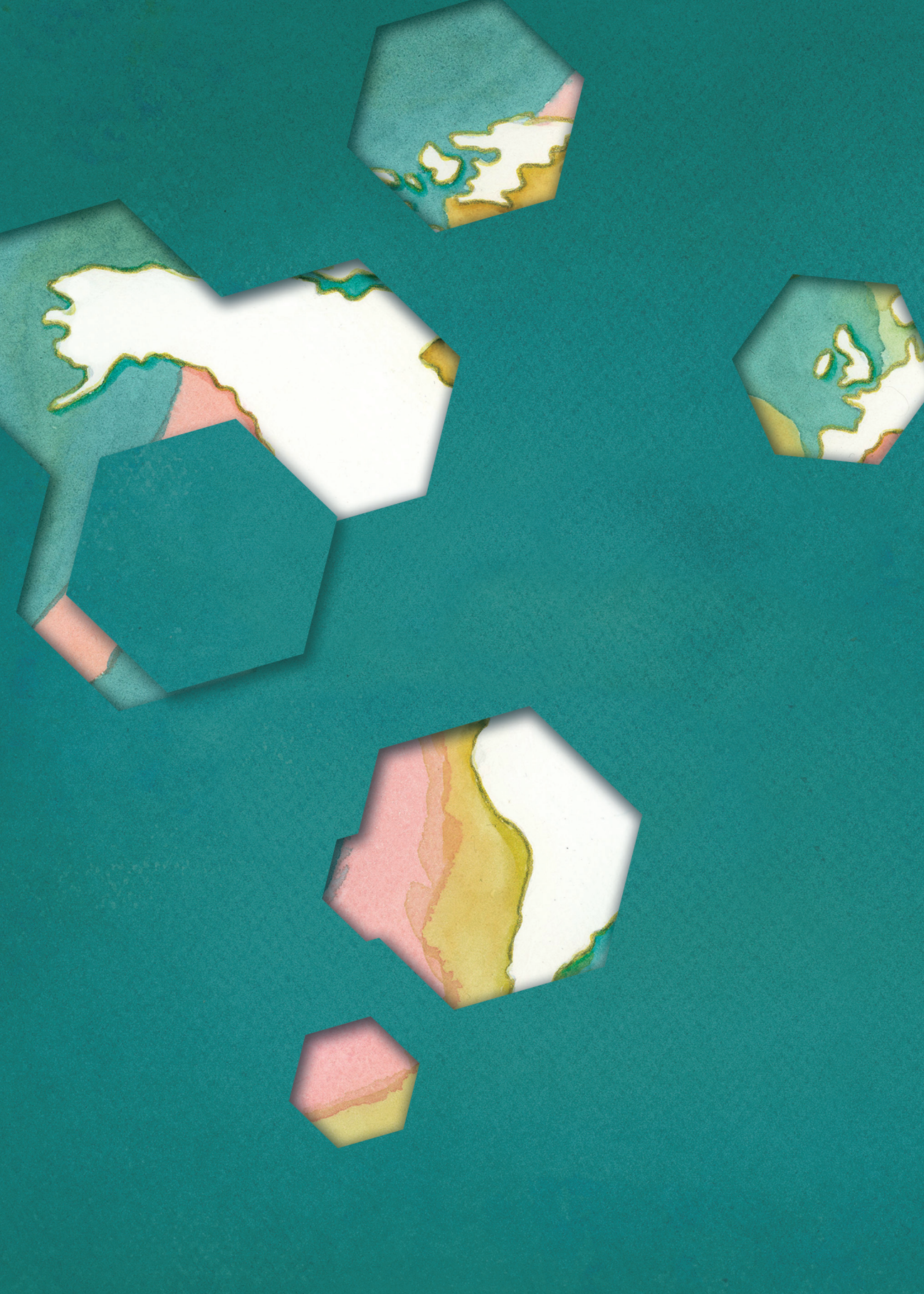


Figure 1. Graphic outline of this thesis.

REFERENCES

1. Itan Y, Powell A, Beaumont MA, Burger J, and Thomas MG. The origins of lactase persistence in Europe. *PLoS Comput Biol* (2009) 5(8): e1000491.
2. Steele JH. History, trends, and extent of pasteurization. *J Am Vet Med Assoc* (2000) 217(2): 175-8.
3. Currier RW and Widness JA. A brief history of milk hygiene and its impact on infant mortality from 1875 to 1925 and implications for today: a review. *J Food Prot* (2018) 81(10): 1713-22.
4. Oliver SP, Boor KJ, Murphy SC, and Murinda SE. Food safety hazards associated with consumption of raw milk. *Foodborne Pathog Dis* (2009) 6(7): 793-806.
5. Rankin SA, Bradley RL, Miller G, and Mildenhall KB. A 100-year review: a century of dairy processing advancements - Pasteurization, cleaning and sanitation, and sanitary equipment design. *J Dairy Sci* (2017) 100(12): 9903-15.
6. Claeys WL, Cardoen S, Daube G, De Block J, Dewettinck K, Dierick K, *et al*. Raw or heated cow milk consumption: review of risks and benefits. *Food Control* (2013) 31(1): 251-62.
7. Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S, *et al*. Food processing and allergenicity. *Food Chem Toxicol* (2015) 80: 223-40.
8. Chandan RC. Dairy ingredients for food processing: an overview in *Dairy Ingredients for Food Processing*. (2011): 3-33.
9. Michalski MC and Januel C. Does homogenization affect the human health properties of cow's milk? *Trends Food Sci Tech* (2006) 17(8): 423-37.
10. Brick T, Ege M, Boeren S, Bock A, von Mutius E, Vervoort J, *et al*. Effect of processing intensity on immunologically active bovine milk serum proteins. *Nutrients* (2017) 9(9): 963.
11. Braun-Fahrlander C and von Mutius E. Can farm milk consumption prevent allergic diseases? *Clin Exp Allergy* (2011) 41(1): 29-35.
12. World Allergy Organisation (WAO). White book on allergy: update 2013. Available from: https://www.worldallergy.org/UserFiles/file/WhiteBook2-2013-v8.pdf Accessed on: 21 June 2019
13. Eder W, Ege MJ, and von Mutius E. The asthma epidemic. *N Engl J Med* (2006) 355(21): 2226-35.
14. Braun-Fahrlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, *et al*. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. *Clin Exp Allergy* (1999) 29(1): 28-34.
15. Kilpelainen M, Terho EO, Helenius H, and Koskenvuo M. Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* (2000) 30(2): 201-8.
16. von Ehrenstein OS, von Mutius E, Illi S, Baumann L, Bohm O, and von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* (2000) 30(2): 187-93.
17. Riedler J, Eder W, Oberfeld G, and Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin Exp Allergy* (2000) 30(2): 194-200.
18. Alfven T, Braun-Fahrlander C, Brunekreef B, von Mutius E, Riedler J, Scheynius A, *et al*. Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle - The PARSIFAL study. *Allergy* (2006) 61(4): 414-21.
19. von Mutius E and Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* (2010) 10(12): 861-8.
20. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and

development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
21. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, *et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
22. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
23. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
24. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
25. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-706 e13.
26. Chatterton DE, Nguyen DN, Bering SB, and Sangild PT. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int J Biochem Cell Biol* (2013) 45(8): 1730-47.
27. Cano-Ruiz M and Richter R. Effect of homogenization pressure on the milk fat globule membrane proteins. *J Dairy Sci* (1997) 80(11): 2732-9.
28. Wijga AH, Smit HA, Kerkhof M, de Jongste JC, Gerritsen J, Neijens HJ, *et al*. Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: the PIAMA birth cohort study. *Thorax* (2003) 58(7): 567-72.
29. Abbring S, Hols G, Garssen J, and van Esch BCAM. Raw cow's milk consumption and allergic diseases - The potential role of bioactive whey proteins. *Eur J Pharmacol* (2019) 843: 55-65.
30. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
31. Melnik BC, John SM, Carrera-Bastos P, and Schmitz G. Milk: a postnatal imprinting system stabilizing FoxP3 expression and regulatory T cell differentiation. *Clin Transl Allergy* (2016) 6: 18.



CHAPTER 2

Raw cow's milk consumption and allergic diseases – The potential role of bioactive whey proteins

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ABSTRACT

The prevalence of allergic diseases has increased significantly in Western countries in the last decades. This increase is often explained by the loss of rural living conditions and associated changes in diet and lifestyle. In line with this 'hygiene hypothesis', several epidemiological studies have shown that growing up on a farm lowers the risk of developing allergic diseases. The consumption of raw, unprocessed, cow's milk seems to be one of the factors contributing to this protective effect. Recent evidence indeed shows an inverse relation between raw cow's milk consumption and the development of asthma and allergies. However, the consumption of raw milk is not recommended due to the possible contamination with pathogens. Cow's milk used for commercial purposes is therefore processed, but this milk processing is shown to abolish the allergy-protective effects of raw milk. This emphasizes the importance of understanding the components and mechanisms underlying the allergy-protective capacity of raw cow's milk. Only then, ways to produce a safe and protective milk can be developed. Since mainly heat treatment is shown to abolish the allergy-protective effects of raw cow's milk, the heat-sensitive whey protein fraction of raw milk is an often mentioned source of the protective components. In this review, several of these whey proteins, their potential contribution to the allergy-protective effects of raw cow's milk, and the consequences of heat treatment will be discussed. A better understanding of these bioactive whey proteins might eventually contribute to the development of new nutritional approaches for allergy management.

INTRODUCTION

Allergic diseases are a global health problem. They affect one billion people worldwide and their prevalence is expected to increase to four billion by 2050 (1). In the EU, the estimated health care costs to manage allergic diseases range between 55 and 151 billion euro (2). Currently, there is neither a cure nor a treatment. Patients should strictly avoid the allergen, but this does not prevent accidental exposures which can have life-threatening consequences.

The rapid rise in the prevalence of allergic diseases is mainly evident in Western countries. This rise is often attributed to a reduced microbial burden in early childhood as a consequence of urbanization (3, 4). In line with this 'hygiene hypothesis', several epidemiological studies have shown a protective 'farm effect' on allergic diseases (5). Studies investigating populations with a similar genetic background but different environment exposures have consistently shown that children growing up on a farm have a lower risk of developing asthma and allergies (6-10). Environmental factors contributing to this protective effect are contact with farm animals, contact with animal feed, stable/barn visits, and the consumption of raw, unprocessed, farm milk (11-15). The latter is of particular interest, since its protective effect was found to be independent of farm-related co-exposures (13-15). This suggests that non-farming populations might equally benefit from the protective effects of raw farm milk consumption. The observed association between raw farm milk consumption and the protection against allergic diseases reported by these epidemiological studies was recently strengthened by the finding of a causal relationship. In a murine model, raw cow's milk consumption was shown to prevent the development of house dust mite-induced asthma (16).

Theoretically, raw cow's milk consumption can thus be considered as a preventive treatment, but in reality the consumption is discouraged because of the possible contamination with pathogens. The most commonly detected pathogens in bulk tank milk are *Listeria*, *Salmonella*, *Campylobacter*, Enterohemorrhagic *Escherichia coli*, and Shigatoxigenic *Escherichia coli* (17, 18). The risk of disease outbreaks that could be caused by these pathogens is the basis for governmental agencies to prohibit the consumption of raw cow's milk, especially for pregnant women, infants, and children (19). Nevertheless, raw cow's milk is still widely consumed by dairy farming families and to a certain extent also by rural non-farming families. When produced under strict hygienic and microbiological standards, the risks of raw cow's milk consumption are rather low. In Germany, for example, raw cow's milk is even sold commercially. This milk, better known as 'Vorzugsmilch', is a legally controlled raw milk certified for consumption (20).

Cow's milk used for commercial purposes is, however, processed. This milk processing ensures microbial safety, but it has also been shown to abolish the asthma- and allergy-protective effects of raw cow's milk (16, 21, 22). This emphasizes the need to elucidate the

raw milk components responsible for the allergy-protective effects and their underlying mechanism, only then ways of producing a safe and protective milk can be developed. Since loss of protection is mainly observed after heat treatment, the heat-sensitive whey protein fraction of raw milk is often mentioned as source of the allergy-protective components. The immunological effects of several of these whey proteins, their potential contribution to the allergy-protective effects of raw cow's milk and the consequences of heat treatment will be discussed in this review.

MILK PROCESSING

The cow's milk most people consume in developed countries is not raw but is extensively processed. This processing consists of various steps in order to preserve milk along the supply chain. Upon collection, which might involve machine milking, milk is cooled and stored at 4 °C. After transport to the dairy plant, the milk is centrifuged in order to remove the milk fat, leaving skim milk. The milk fat and skim milk will be recombined in the desired ratios to obtain: skimmed milk (≤ 1% fat), semi-skimmed milk (2% fat) or whole milk (> 3.25% fat) (23). After this standardization process, the milk is heat-treated. Based on heating time and temperature, different heat treatments can be distinguished. The most commonly used heat treatments are pasteurization (71-74 °C for 15-40 s), sterilization (110-120 °C for 10-20 min), and ultra-high temperature (UHT) processing (135-145 °C for 0.5-4 s) (18, 24). Heating inactivates pathogenic microorganisms and is used to maintain microbial safety of the milk. The effectiveness of heat treatment is determined by measuring alkaline phosphatase activity. Alkaline phosphatase is an enzyme naturally present in raw milk. The heat resistance of alkaline phosphatase is slightly higher than that of the most heat-stable bacterium found in raw milk, *Mycobacterium paratuberculosis*. This makes the enzyme an ideal indicator of product safety (25). Heat treatment is often followed by homogenization, although homogenization may also take place prior to heat treatment. During homogenization the milk is pumped under high pressure through narrow pipes. This reduces the size of the fat globules and thereby it prevents the separation of a cream layer. Homogenization increases the stability of the milk resulting in an increased shelf life (26). After heating and homogenization, the milk is rapidly cooled to 4 °C and is subsequently packaged and stored for commercial purposes.

From all these processing steps, mainly homogenization and heat treatment are thought to affect the allergy-protective capacity of raw milk. Homogenization results in profound changes in the milk fat structure. Reducing the size of the fat globules will largely increase the total droplet surface area. To cover this increase in surface area, milk proteins will be included. These milk proteins will mainly be caseins and to a minor extent also whey proteins (β-lactoglobulin, α-lactalbumin) (27). Since caseins are one of the main milk allergens, homogenization is thought

to affect allergen presentation to the immune system. Animal models show that this altered allergen presentation favors milk allergy (28, 29), but these findings could not be confirmed in clinical studies (30, 31). Since current evidence mainly indicates a loss of protection after heating the milk, this review will focus on the consequences of heat treatment on raw milk components rather than on the consequences of homogenization. For further reading about the possible effects of homogenization on the health properties of milk, we refer to a review by Michalski (32).

Heating will mainly affect heat-sensitive milk components, such as proteins. Cow's milk consists for about 3.5% of proteins. Approximately 82% of these proteins are caseins and 18% are whey proteins. The casein protein family consists of αs1-, αs2-, β-, and κ-casein whereas the whey protein family consists of α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulins, and many minor proteins and enzymes (33). Caseins are heat-stable and are therefore not affected by heat treatment. However, whey proteins are heat-sensitive and heating will cause denaturation, aggregation, and glycation of these proteins. Such processes might structurally alter the whey proteins and thereby they might (at least partly) lose functionality (34). Since many of the whey proteins have immunomodulatory capacities, denaturation of these by heating is often hypothesized to abolish the allergy-protective effects of raw cow's milk.

BIOACTIVE WHEY PROTEINS

Dietary bioactive components are defined as 'food components that can affect biological processes or substrates and hence have an impact on body function or condition and ultimately health' (35). The whey protein fraction of raw cow's milk contains many of these so-called 'bioactive components'. Although the major whey proteins α-lactalbumin, β-lactoglobulin, and bovine serum albumin do not have immune-related functionalities that can directly be linked to the protective effects of raw cow's milk, several less abundant whey proteins do (36). A complete overview of these bioactive whey proteins is beyond the scope of this review. Here, the focus is on a selected set of bioactive components often mentioned in relation to the allergy-protective capacity of raw cow's milk (**Table 1**).

Immunoglobulins

Together with lactoferrin, lactoperoxidase, and lysozyme, immunoglobulins form the antimicrobial system of bovine milk. They provide the newborn with immunological protection against microbial infections and confer passive immunity until the newborns own immune system has fully matured (37). The predominant immunoglobulin in bovine milk is IgG, but IgA and IgM are also present (38).

A direct link between IgG and allergies was observed in a murine model where IgG protected against ovalbumin (OVA)-induced asthma by forming immune complexes. These immune complexes of allergen and allergen-specific IgG, found in breast milk, were taken up via the neonatal Fc receptor resulting in oral tolerance to the allergen by the induction of $FoxP3^{+}CD25^{+}$ regulatory T cells (39). Whether bovine milk IgG can also confer protection against allergic diseases by forming immune complexes with allergens has never been studied. However, it is shown that bovine milk contains IgG antibodies specific for human allergens, like house dust mite, *Aspergillus* species, grass pollen and birch pollen (38, 40). These allergens might also be present in small amounts in bovine milk or they can be ingested/inhaled (a part of inhaled allergens will be cleared from the upper airways and will be swallowed) simultaneously. Upon concurrent ingestion of bovine milk and allergenic proteins immune complexes might be formed (38). Since bovine IgG is shown to have some affinity for the human neonatal Fc receptor, which is expressed in the human intestine, this might theoretically be a way by which oral tolerance can be induced (41).

Interestingly, bovine IgG was also shown to bind to a wide range of human pathogenic bacteria and viruses (42-44). It binds for example to human respiratory syncytial virus (RSV) and enhances its phagocytosis via FcγRII receptors on macrophages, neutrophils, and monocytes (45). Although this might not directly link to the observed allergy-protective effects of raw cow's milk consumption, RSV infection in childhood is associated with the development of asthma later in life (46, 47). One might therefore speculate that RSV-specific IgG antibodies present in bovine milk could contribute to the asthma-protective capacity of raw cow's milk consumption.

In addition, allergen-specific IgG antibodies are known to exert a strong suppressive effect on IgE-mediated activation of mast cells and basophils (48). They can counteract the effects of IgE via two mechanisms; IgG-mediated blocking and receptor-mediated inhibition. In the first mechanism, specific IgG antibodies bind to allergens before these allergens encounter mast cells. Thereby they mask the IgE-binding epitopes on the allergen which prevents binding of the allergen to IgE (49). In the second mechanism, the allergen simultaneously binds to IgE and IgG antibodies present on the surface of mast cells which induces cross-linking of their receptors (high affinity FcεRI and low affinity FcγRIIb respectively). Since signaling via the inhibitory IgG receptor, FcγRIIb, counteracts IgE-mediated mast cell activation, this negatively regulates the allergic response (50-52). The negative regulation of IgE-mediated allergic reactions by the concurrent IgG response to the same antigen is thought to be involved in the natural resolution of food allergies with age and in oral immunotherapy (regular oral administration of increasing doses of allergen to acquire unresponsiveness) (49). Whether bovine milk-derived (allergen-specific) IgG antibodies are also capable of suppressing allergic responses and whether this contributes to the allergy-protective capacity of raw cow's milk has never been studied.

Lactoferrin

Lactoferrin is produced and released by mucosal epithelial cells into most exocrine fluids, and particularly into milk (53). It is an iron-binding glycoprotein with many functionalities from which the protection against microbial pathogens was the first one discovered. This antimicrobial activity of lactoferrin is due to two different mechanisms. The first mechanism relates to its iron scavenging function in the intestine. By binding iron, lactoferrin reduces the availability of free iron required by iron-dependent pathogens and thereby it inhibits their growth. The second mechanism involves a direct interaction of lactoferrin with the bacterial cell wall. Lactoferrin binds to the lipid A portion of LPS on the bacterial cell surface resulting in destabilization of the cell membrane and bacterial cell lysis (54, 55). Lactoferrin not only has antimicrobial properties, it also has immunomodulatory effects. It for example inhibits the production of pro-inflammatory cytokines such as TNF-α and IL-1β by binding to pathogen-associated molecular patterns (PAMPs) like LPS and CpG-containing DNA. Such binding was shown to hamper LPS signaling and to inhibit LPS-induced activation of immune cells. Lactoferrin can also act as chemoattractant for immune cells, it can act as antioxidant due to its iron-binding capacity, it can affect epithelial cell growth and maturation, and it can furthermore modulate cell-mediated and humoral immune responses by promoting the maturation, differentiation, and activation of T- and B-lymphocytes (56-58).

At first glance, these functionalities seem to be primarily linked to host defense against infections and excessive inflammation. However, some of these functions might also indirectly relate to the prevention of allergic diseases. The iron scavenging function of lactoferrin in the intestine for example, does not only prevent the growth of iron-dependent pathogens, it also promotes the growth of bacteria with low iron requirements such as *Bifidobacteria* and *Lactobacilli* (54, 59, 60). These bacteria are considered to be beneficial to the host and their presence in the gut microbiota of infants seems to correlate with protection against allergic diseases (61-63). Besides, they are known to be potent producers of short-chain fatty acids (SCFA) by fermenting non-digestible oligosaccharides in the colon. From these SCFA (e.g. butyrate, acetate, and propionate) it is known that they can prevent the development of allergies by enhancing epithelial integrity, inhibiting mast cell activation, and promoting regulatory T cell differentiation and IgA release from plasma cells (64-66). A direct link between lactoferrin and allergic diseases is, to our knowledge, only investigated by one study. Kruzel *et al*. showed that lactoferrin reduces pollen antigen-induced airway inflammation in a murine asthma model (67). By binding free iron, lactoferrin lowered the ragweed pollen extract-induced increase in cellular reactive oxygen species levels in bronchial epithelial cells and thereby it decreased the accumulation of inflammatory cells in the airways.

Even though bovine lactoferrin has only 77% sequence homology with human lactoferrin at the mRNA level and only 69% at the protein level it is shown to be taken up by the human intestinal lactoferrin receptor (68, 69). This suggests that bovine lactoferrin exerts several of the biological activities of human lactoferrin. Indeed, in human colon epithelial cells it has been shown that bovine lactoferrin increased cell proliferation, enhanced cell differentiation, and stimulated the expression of TGF-β (70). Moreover, human monocyte-derived dendritic cells (DCs) differentiated in the presence of bovine lactoferrin showed a tolerogenic phenotype (71).

Bovine lactoferrin has already been shown to be protective against respiratory tract infections in infants when added to infant formula (72). Whether it also protects infants from developing allergic diseases has never been studied. However, its probiotic effects together with its immunomodulatory capacity makes lactoferrin a promising allergy-protective raw milk ingredient.

TGF-β

Next to bovine IgG and lactoferrin, TGF-β has also been linked to immunological effects that can contribute to the allergy-protective capacity of raw cow's milk. TGF-β consists of five isoforms (β1-β5), of which TGF-β1 and TGF-β2 can be found in bovine milk. From these two, TGF-β2 is most abundantly present and has a 100% sequence homology with its human counterpart suggesting a similar physiological effect of both forms (73). Besides from being present in milk, TGF-β is also endogenously produced. Many cell types, including intestinal epithelial cells and immune cells (like T cells, B cells, DCs, and macrophages) can produce the TGF-β1 isoform (74).

TGF-β is a multifunctional cytokine which plays a key role in the development and maturation of the mucosal immune system (75). Besides, TGF-β1 is known to enhance epithelial differentiation and intestinal barrier function. It, for example, increased the transepithelial electrical resistance of human colon-derived epithelial monolayers and prevented epithelial barrier disruption caused by exposure to IFNγ or by infection with Enterohemorrhagic *Escherichia coli* or *Cryptosporidium parvum* (76-78). These protective effects were, at least partially, induced by increased expression of intestinal tight junctions leading to improved barrier function (79). The capacity of TGF-β1 to enhance intestinal barrier function provides a possible link with allergic diseases. Food allergies, for instance, are shown to be associated with altered intestinal epithelial barrier function. A breakdown in intestinal barrier function increases the exposure of dietary allergens to the mucosal immune system, leading to allergic sensitization and subsequently the production of allergen-specific IgE antibodies (80). The effect of TGF-β1 on intestinal barrier function could explain the observed protection of TGF-β against allergies in infancy and early childhood (75).

Another explanation for the protective effect of TGF-β on allergic diseases arises from its ability to induce and maintain oral tolerance. Oral tolerance is a state in which the immune system, locally as well as systemically, does not respond to generally harmless antigens, such as food proteins. This phenomenon specifically takes place in the gut and develops upon oral dietary antigen exposure. Oral tolerance induction is thought to occur through several mechanisms including anergy or deletion of antigen-specific T cells and active cellular suppression by regulatory T cells. Defective oral tolerance to innocuous food proteins is suggested to result in food allergy (81). The crucial role of TGF-β in the induction of immune tolerance is illustrated by, for example, Verhasselt *et al*. who demonstrated in a murine model that the presence of TGF-β is required to induce oral tolerance to allergens present in breast milk in the absence of specific IgG (82). In agreement with these studies, Penttila *et al*. showed that formula milk without TGF-β induced a proinflammatory cytokine profile together with increased numbers of activated mast cells, eosinophils, and DCs in the gut of rat pups (83). Supplementation with physiological amounts of TGF-β induced oral tolerance to the cow's milk protein β-lactoglobulin by shifting the immune response from a Th2 to a Th1 profile. In addition, TGF-β supplementation resulted in an increased IL-10 production. Downregulation of the allergic response was maintained when TGF-β was no longer present in the diet (84).

One of the ways by which TGF-β is thought to induce tolerance is by inducing different subclasses of regulatory T cells. Regulatory T cells are critical for developing and maintaining oral tolerance in the gut. The majority of regulatory T cells is dependent on the transcription factor FoxP3 for their development. These $FoxP3^+$ regulatory T cells are mainly generated in the thymus, but they can also be induced in the periphery from naïve T cells. TGF-β appears to play a key role here, as it was shown to induce FoxP3 expression and to convert naïve peripheral T cells into $FoxP3^+$ regulatory T cells (85). This conversion could not take place when TGF-β signaling was deficient, showing the necessity of TGF-β induced FoxP3 expression (86). Besides inducing $FoxP3^+$ regulatory T cells, TGF-β also induces regulatory T cells subsets producing IL-10 and TGF-β (Tr1 and Th3 cells respectively) (87, 88). The importance of the latter was demonstrated in a study showing reduced numbers of TGF-β-producing regulatory T cells in the intestine of food allergic children (89).

Another way by which TGF-β contributes to oral tolerance induction is via its capacity to induce IgA class switching in B cells. IgA is the predominant class of immunoglobulin present in intestinal secretions. It prevents adhesion of bacteria and viruses to mucosal epithelial cells and maintains tolerance to commensal bacteria. Just like IgG, IgA can trap food allergens, preventing them from binding to IgE (88). In epidemiological studies, IgA deficiency was associated with infections and allergic diseases during childhood (90). In addition, low levels of human milk IgA correlated with allergy development (91, 92). These studies indicate a potential role of IgA in oral tolerance development.

Possibly together with other raw milk components, TGF-β creates a regulatory environment (inducing regulatory T cell development and IgA production) which favors unresponsiveness upon allergen exposure. Interestingly, there also seems to be a positive feedback loop between TGF-β production in the gut and other raw milk components such as lactoferrin. It has been shown that lactoferrin stimulates the production of TGF-β by intestinal epithelial cells, stressing the importance of TGF-β presence in the gut (68, 70). The strong tolerogenic capacity of TGF-β makes it a promising candidate that may underlie the allergy-protective effects of raw cow's milk consumption.

IL-10

Just like TGF-β, IL-10 is a regulatory cytokine present in bovine milk although in much lower concentrations (38). It is a pleiotropic cytokine with many functionalities relevant to allergic diseases. Generally, IL-10 conditions the gut to be a tolerogenic environment. More specifically, it modulates Th2 responses associated with allergic diseases (93). It, for example, inhibits IgE-induced mast cell activation, Th2 cell activation, and eosinophil function (94-96). It also inhibits antigen-presenting cell (APC) function by reducing the expression of MHCII and co-stimulatory molecules and by preventing DC maturation (97, 98). IL-10 furthermore enhances immunoglobulin class switching in B cells and it possibly induces IL-10-secreting regulatory T cells (99, 100). In line with these findings, there is evidence for an inverse correlation between IL-10 and allergic diseases (101, 102).

Bovine IL-10 appears to have a 76.8% amino acid sequence homology with human IL-10. In addition, it was shown to bind to the human IL-10 receptor (103). This indicates that bovine IL-10 might exert immunomodulatory activities on human immune cells. Bovine IL-10 indeed showed to inhibit LPS-induced human DC activation. The expression of DC activation markers, CD40, CD80, and CD86, was dose-dependently reduced by bovine IL-10 and the production of IL-12, TNF-α, and IL-1β was inhibited. Similar results were observed for human monocytes (103). Bovine IL-10 was furthermore shown to be equally effective in inhibiting human DC activation as human IL-10 (103). In the presence of TGF-β and IL-10, DCs with a low expression of the co-stimulatory molecules CD80 and CD86 can convert naïve peripheral T cells into $FoxP3^+$ regulatory T cells (88, 104).

As mentioned earlier, the anti-inflammatory cytokines present in raw cow's milk (e.g. IL-10 and TGF-β) could be essential for the induction of an environment favoring tolerance towards allergens. The endogenous production of these anti-inflammatory cytokines is moreover stimulated by other raw milk components, like lactoferrin. Bovine milk lactoferrin was shown to enhance the release of IL-10 by intraepithelial lymphocytes in the gut (a similar effect was observed for TGF-β, as described earlier) (105). The tolerogenic feature of these cytokines could contribute to the observed allergy protection by raw cow's milk consumption.

Alkaline phosphatase

Alkaline phosphatase (ALP) is an enzyme naturally present in raw milk of all mammalian species. It is probably best known for its role in dairy industry as an indicator of successful pasteurization. Upon pasteurization of raw milk, ALP becomes inactivated and loses its activity. Consequently, levels are low in processed milk and milk products (25). Next to its presence in raw milk, ALP is also produced endogenously. It is ubiquitously distributed among cell types and tissues. Four distinct ALP isoforms exist; tissue non-specific ALP (the predominant circulating form, located and expressed mainly in bone, liver and kidney), placenta ALP, germ cell ALP, and intestinal ALP (106). The tissue non-specific ALP isoform is present in raw milk (107). About 30% of this ALP is bound to the fat fraction, while the remaining part is in the milk serum (108).

From the various ALP isoforms, intestinal ALP is the most studied. Intestinal ALP is secreted by enterocytes and has many biological functions. It for example regulates duodenal bicarbonate secretion and surface pH, it modulates intestinal long-chain fatty acid absorption, it reduces intestinal translocation of bacteria, and it detoxifies bacterial LPS by dephosphorylation of the lipid A moiety (106). The latter makes intestinal ALP a potential therapeutic agent for LPS-mediated diseases. Several studies have already demonstrated that exogenous intestinal ALP administration effectively reduces inflammatory diseases such as inflammatory bowel disease, necrotizing enterocolitis, and sepsis (109-111). Whether the tissue non-specific ALP isoform present in bovine raw milk is also able to detoxify LPS leading to anti-inflammatory effects is not documented yet. However, since human tissue non-specific ALP does prevent LPS-induced sepsis in mice this is very likely (112).

Whether exogenous ALP administration (for example via raw cow's milk consumption) can also affect allergic diseases has, to our knowledge, never been studied. One could argue that ALP might impact food allergy, since it reduces inflammatory responses by detoxifying bacterial LPS, which could prevent gut permeability. In addition, there is some indication that ALP levels are reduced in cow's milk allergy, just as has been shown for the inflammatory diseases mentioned earlier (110, 111, 113). This could suggest that consuming raw cow's milk, as source of ALP, could be beneficial. However, evidence is poor and these are speculations, which should be confirmed in future research.

Osteopontin

Another bioactive whey protein recently gaining interest is osteopontin (OPN). OPN is an extensively phosphorylated glycoprotein that is synthesized by various tissues and is present in most body fluids, including milk. Although an intracellular form has been described, OPN is primarily a secreted protein which exerts its functions through binding to cell surface integrins or to the CD44 receptor. It is involved in many physiological and pathological processes

such as biomineralization, tissue remodeling, tumorigenesis, and cellular adhesion, migration, and survival (114). OPN is encoded by one gene but several isoforms exist as a result of posttranslational modifications. These modifications vary greatly between cell types and are responsible for the diverse biological functions of OPN (115).

OPN is expressed by many immune cells, such as T cells, B cells, DCs, macrophages, and mast cells. It was first described as a Th1 cytokine since it contributes to the development of Th1-mediated immune responses and diseases. OPN for example activates DCs and polarizes them towards a Th1-promoting phenotype (116). In addition, OPN-deficient mice show insufficient Th1 immunity when infected with *Mycobacterium bovis* BCG (117). Levels of OPN were furthermore found to be elevated in several Th1-associated diseases, such as rheumatoid arthritis, multiple sclerosis, Crohn's disease, and tuberculosis (118-121).

Since Th1 and Th2 immune responses are often reciprocally regulated, OPN was thought to also modulate allergic responses via its Th1-promoting activity. Indeed, OPN expression was shown to be upregulated in subjects who showed decreased venom-specific IgE levels after successful venom allergen immunotherapy (122). In addition, OPN knockout mice showed significant higher levels of OVA-induced IgE and systemic Th2 responses than wild-type mice in a model for systemic allergic sensitization. These OVA-IgE levels were furthermore dampened after administration of recombinant OPN (123). However, there is also evidence showing that OPN can enhance allergic responses. In *in vitro* cultured mast cells, OPN increased IgE-induced mast cell degranulation and in a murine model of OVA-induced allergic airway inflammation, OPN neutralization before primary sensitization diminished the allergic response upon re-challenge (124, 125). Interestingly, when OPN was neutralized before secondary challenge, the allergic response was exaggerated. This dual role of OPN was attributed to differences in the recruitment of DC subsets. OPN prevented migration of Th2-suppressing plasmacytoid DCs to the draining lymph nodes during sensitization and suppressed migration of Th2-promoting conventional DCs to the lymph nodes during challenge (125). A relation between OPN and allergic diseases was furthermore demonstrated by recent findings showing increased OPN expression in Th2-related diseases, like asthma (126).

Different studies have shown contradictory roles of OPN in allergic diseases, possibly due to its complicated structure with multiple isoforms showing different biological functions. Only the highly phosphorylated, full-length, isoform of OPN is found in bovine milk. The phosphorylation extent of this isoform is much higher than that of any of the endogenously produced OPN isoforms, which can result in different functionalities (127). All previously mentioned studies have focused on the role of endogenous OPN on allergic diseases. Whether orally ingested OPN affects allergic diseases has not yet been explored. Interestingly, milk OPN shows a high affinity for lactoferrin. It has therefore been suggested that OPN might act as a carrier

protein for lactoferrin in milk, protecting lactoferrin from proteolysis upon ingestion (128). This illustrates that besides a possible direct allergy-protective effect, OPN can also indirectly influence allergic outcomes.

Table 1. Proposed allergy-protective effects of (a selected set of) bioactive whey proteins present in raw cow's milk.

Bioactive whey protein	Potential allergy-protective functionalities	Reference
IgG	• Allergen-IgG immune complexes	(38-41)
	• IgG-mediated blocking	(48, 49)
	• Receptor-mediated inhibition	(50-52)
	• Bacteria and virus clearance	(42-44)
Lactoferrin	• Antimicrobial activity	(54, 55)
	• Outgrowth of *Bifidobacteria* and *Lactobacilli*	(54, 59, 60)
	• Stimulation of TGF-β and IL-10 production in the gut	(68, 70, 105)
TGF-β	• Improvement of intestinal barrier function	(76-79)
	• Induction of different subclasses of regulatory T cells (FoxP3$^+$ regulatory T cells, Tr1 cells, Th3 cells)	(85-88)
	• IgA class switching	(88)
IL-10	• Inhibition of APC function	(97, 98)
	• Inhibition of mast cell activation, Th2 cell activation and eosinophil function	(94-96)
	• IgG class switching	(99)
	• Induction of Tr1 cells	(100)
Alkaline phosphatase	• Detoxification of bacterial LPS	(106)
Osteopontin	• Modulation of Th1/Th2 immune responses?	(116, 117, 122-125)
	• Carrier protein lactoferrin	(128)

APC, antigen-presenting cell; Tr1, type 1 regulatory T cell.

MICROBIAL COMPOSITION

Besides the heat-sensitive whey protein fraction, the microbial load of raw milk is another often mentioned factor that could be responsible for the observed protective effects. Especially when the effects are indeed related to the heat treatment of milk, the contribution of bacteria must be considered.

Several studies have shown that the microbial composition of raw and pasteurized milk differs significantly (27, 129). Raw milk was found to contain more bacteria, but also higher levels of bacterial endotoxins compared to pasteurized milk (130, 131). These endotoxins, such as LPS, are structural components of bacteria which can induce immunological responses. They are hypothesized as one of the mechanisms by which the farming environment can be allergy

protective (132). However, this hypothesis is mainly based on the inhalation rather than on the ingestion of endotoxins. The analysis of endotoxin levels in raw milk is usually performed in samples taken from a bulk milk tank on a dairy plant. Since different handling and storage conditions are known to affect the LPS content of the milk, little is known about the levels in milk as it is consumed by farming families in everyday life (131). Gehring *et al*. therefore measured LPS levels in milk samples collected from the homes of farming and non-farming families. Surprisingly, they did not observe any differences in endotoxin levels between raw milk and commercial milk (133). To date, evidence for a protective effect of raw milk endotoxin is inconclusive.

In the GABRIELA study, associations between objectively studied raw milk components and asthma and atopy were investigated. As observed by several other studies, viable bacterial cell counts were elevated in raw milk compared to processed milk samples. However, these bacterial cell counts were not associated with asthma and atopy suggesting that the microbial composition does not contribute to the allergy-protective effect of raw cow's milk consumption (21). In addition, raw milk contains many antimicrobial components, such as lactoferrin, lactoperoxidase, and lysozyme, making the involvement of bacteria less likely.

Next to bioactive whey proteins and bacteria, also fatty acids, oligosaccharides, vitamin A, and vitamin D are thought to contribute to the allergy-protective capacity of raw cow's milk. However, since all these components are heat-stable they are not discussed in this review. In addition, microRNAs are sometimes mentioned as beneficial raw milk ingredient. For further reading about the potential allergy-protective effects of these raw milk-derived microRNAs we refer to an excellent review by Melnik *et al*. (134).

PASSAGE THROUGH THE GASTROINTESTINAL (GI) TRACT

To be able to suppress allergic responses, bioactive whey proteins in raw cow's milk must be able to survive through the upper GI tract. During this passage they will encounter different pH levels and digestive enzymes. Depending on the sensitivity of the proteins, this can hamper their intact arrival in the gut. On the other hand, there are also whey proteins that can be activated by the conditions in the GI tract.

An example of such a whey protein is TGF-β. In milk, TGF-β is mainly associated with latency-associated peptide (LAP) (75). This latent form of TGF-β requires activation (removal of LAP protein) to be able to exert its biological activity. This activation can be triggered by for example α_v integrins, thrombospondin-1, and reactive oxygen species but also by proteases and low pH

(135). Passage through the stomach might therefore activate the latent form of TGF-β present in milk. That orally administered TGF-β can be biologically active in the intestinal mucosa is demonstrated by several animal studies. These studies show that TGF-β retained sufficient activity to enhance oral tolerance to dietary allergens (83, 136). Comparable results have been observed for IL-10 (137).

Even though lactoferrin is not activated by the conditions in the GI tract, it does largely withstand the acidic environment of the stomach. Significant amounts of orally administered bovine lactoferrin were shown to survive passage through the stomach in adults (138). In exclusively breast-fed infants, a substantial proportion of lactoferrin was found in their stool and concentrations of lactoferrin in these fecal samples decreased when levels in breast milk decreased (139). The biological potential of lactoferrin might even be greater in infants than in adults due to their milder digestion (higher gastric pH, lower protease levels) (140). Gastric hydrolysis of lactoferrin was found to be 20-fold higher in weaning than in suckling rats and luminal degradation of lactoferrin in the small intestine increased substantially after weaning (141). Consumed as part of raw milk, lactoferrin might also be (partly) protected against digestion by the milk matrix. Other milk components, such as OPN, have been shown to protect lactoferrin from proteolysis, increasing the likelihood of its intact arrival in the gut (128). In addition, it is worth mentioning that also the peptides derived from the (limited) proteolysis of lactoferrin, which could be produced in the intestinal lumen after oral digestion, are shown to have biological activity (54).

The survival of bovine IgG through the GI tract has been subject of several studies. In general, IgG is thought to be less susceptible to digestion than other dietary proteins. Numerous clinical studies in humans have illustrated that a significant amount of orally ingested bovine IgG is recovered intact and immunologically active from the ileum and feces (142). However, the recovery rate varies a lot between studies, from trace amounts up to 50%. Just as for lactoferrin, IgG recovery was found to be higher in infants than in adults due to their higher gastric pH and lower rate of proteolysis in the GI tract (143). When IgG antibodies are subjected to proteolytic enzymes, they are degraded to Fc and Fab fragments. These Fab fragments have been shown to retain allergen binding and neutralizing activity as long as they are not denatured (142). However, for Fc-receptor dependent functionalities, IgG needs to remain intact. The fact that bovine IgG is relatively stable to proteolytic digestion (even more stable than human IgG), makes it plausible that milk-derived IgG can also still execute these functionalities (144).

Oral administration of bovine ALP is shown to be protective in experimental models for inflammatory diseases (145). Many of these models focus on intestinal inflammation, suggesting that bovine ALP can withstand the harsh conditions in the GI tract (110, 111, 146). In an animal

model for colonic inflammation, oral administration of ALP was shown to be less effective in reducing colitis than intrarectal administration, suggesting that ALP may have partially degraded in the GI tract (147). However, since oral ALP was still effective, sufficient ALP must be retained to induce this protective effect. This indicates that orally ingested milk-derived ALP has the potential to modulate intestinal immune responses.

Milk OPN is also partly resistant to digestion. A fraction of ingested OPN will therefore reach the intestine and can bind to OPN receptors. Besides intact OPN, also partly digested OPN and OPN peptides were shown to be able to bind to OPN receptors and exert biological activities. In addition, these partially digested OPN forms were shown to be absorbed and to enter the systemic circulation where they can reach other target cells (127). Just as demonstrated for lactoferrin, this indicates that partial digestion of OPN is not necessarily detrimental to its biological functions.

EFFECT OF HEATING ON BIOACTIVE WHEY PROTEINS

Current evidence mainly points towards a loss of allergy protection after heating raw milk, suggesting the importance of heat-sensitive milk components (16, 21, 22). Of all raw milk components, mainly whey proteins are susceptible to heat treatment (148). As described, many of these whey proteins have immune-related functionalities that can be linked to the allergy-protective capacity of raw cow's milk. Losing these functionalities by heating raw milk might therefore be detrimental to the allergy-protective effects.

In general, heating of whey proteins results in their denaturation, aggregation, and glycation which consequently leads to a loss of biological functionality (148). At which temperature this happens depends on the protein, but generally it is assumed that whey proteins denature above 65 °C (24). From the different immunoglobulins present in bovine milk, IgM is the most heat-sensitive, followed by IgA and IgG. Pasteurization at 72 °C for 15 s was shown to denature 14% of IgM, 2% of IgA, and only 1% of IgG, while sterilization and UHT processing completely denatured all immunoglobulins (149, 150). This suggests that a large proportion of milk immunoglobulins, especially of IgG, is retained after pasteurization. However, it should be mentioned that a higher loss of IgG after pasteurization (around 20-40%) has also been reported (151). This is more comparable to results observed in human milk, where IgG levels decreased with about 60% after pasteurization (152). In addition, when measured in commercially available pasteurized milk total IgG levels were much lower compared to raw milk (21, 151).

Bovine lactoferrin starts to denature at 70 °C. At this temperature, an irreversible loss of the secondary structure of lactoferrin was observed. Interestingly, this only occurred when lactoferrin was gradually submitted to increasing temperatures. When lactoferrin was rapidly submitted to a temperature of 72 °C for a short period of time, to mimic the pasteurization process, the secondary structure remained intact (153). On the other hand, during this pasteurization condition the tertiary structure of lactoferrin was affected. Pasteurization may therefore lead to a non-native but also not completely denatured (partially folded) lactoferrin conformation. Possibly because of the intact secondary structure, Zhang *et al.* showed only a small reduction in lactoferrin levels upon pasteurization (154). This small, but significant, reduction was confirmed in commercially available pasteurized shop milk, which contained lower lactoferrin levels than raw milk (155). Higher heat treatments (above 80 °C), such as UHT, substantially lowered lactoferrin levels (148).

Little is known about the denaturation kinetics of bovine milk TGF-β and IL-10. However, just as for IgG and lactoferrin, bovine TGF-β1 levels were found to be lower in commercial pasteurized milk than in raw milk (156). TGF-β2 concentrations did not differ between pasteurized and raw milk, but levels were reduced when raw milk was heated to 87 °C (21, 157, 158). Similar results were obtained for the TGF-β2 content of the whey protein fraction of milk; levels decreased as heat treatment increased in intensity (159). The knowledge for bovine milk IL-10 is even more limited, but the effect of heat treatment on human milk IL-10 is extensively studied. Several studies have shown that human milk IL-10 concentrations were significantly reduced upon pasteurization (160, 161). Since human and bovine IL-10 have a high sequence homology and a comparable functionality, heating might have a similar effect on bovine IL-10.

ALP is used as an indicator of successful pasteurization, it is therefore obvious that it is affected by heat. Pasteurization of bovine milk lowers ALP levels to below the detection limit and will thereby destroy its immune-modulating potential (25). In contrast to ALP, OPN is relatively heat-stable. Pasteurization of bovine milk did not affect OPN concentrations (154). Whether higher heat treatments affect bovine milk OPN is to our knowledge never investigated, but isolated OPN was shown to be stable under a wide range of temperatures, up to 120 °C (128).

The effect of heat treatment on β-lactoglobulin is perhaps the most interesting one. B-lactoglobulin is the most abundant whey protein in cow's milk. Even though β-lactoglobulin has no clear immune-modulating properties that can be lost upon heating, it has a major influence on the biological activity of other whey proteins. Upon denaturation, β-lactoglobulin loses its secondary and tertiary structures and a previously hidden free thiol group (-SH group) becomes exposed. At temperatures above 70 °C this free thiol group reacts with other whey proteins causing irreversible aggregation reactions (162). These heat-induced aggregation reactions are likely to occur with TGF-β2 since this molecule also contains a free thiol group.

In addition, TGF-β2 has a strong hydrophobic character, favoring its polymerization and its interaction with other proteins (159). Moreover, β-lactoglobulin also readily forms aggregates with immunoglobulins (73). These aggregation reactions significantly affect the biological functionality of the whey proteins involved.

CONCLUDING REMARKS

The interest in the allergy-protective effects of raw cow's milk has increased enormously in recent years. The existing epidemiological evidence is lately strengthened by causality and the contribution of heat-sensitive raw milk components seems to be evident. This review focused on the potential role of bioactive whey proteins in the allergy-protective effects of raw cow's milk. A selected set of these proteins (IgG, lactoferrin, TGF-β, IL-10, ALP, and OPN), often mentioned in relation to the allergy-protective effects of raw cow's milk, is discussed in detail. These components were shown to be involved in creating a tolerogenic environment (e.g. promoting regulatory T cell development, inducing IgA production, modulating the gut microbiome, and enhancing epithelial barrier function) which favors unresponsiveness upon allergen exposure (**Figure 1**). Heating clearly affects the concentration as well as the functionality of the whey proteins discussed in this review (except for OPN). The detrimental effects are particularly evident when cow's milk is heated at high temperatures, such as during sterilization or UHT processing. Although pasteurization does not destroy the biological functionality of all whey proteins, for some of them it does. In addition, the temperature used during pasteurization denatures β-lactoglobulin. Since denatured β-lactoglobulin attacks and thereby inactivates other, less heat-sensitive, whey proteins (such as TGF-β2), pasteurization can still be detrimental to the allergy-protective effects of raw cow's milk. Future research should focus on ways to develop a milk which is both safe and allergy protective. For this, a better understanding of the raw milk components responsible for the observed allergy-protective effects and their underlying mechanisms is crucial.

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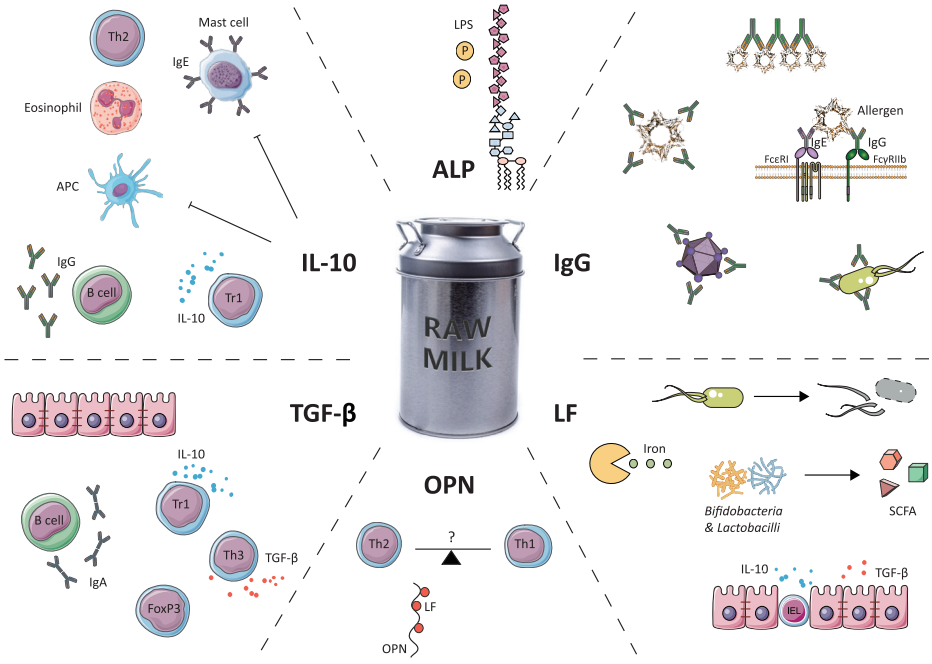


Figure 1. Graphical representation of the potential allergy-protective effects of raw milk-derived bioactive whey proteins. Shown are whey proteins often mentioned in relation to the allergy-protective effects of raw cow's milk. Bovine IgG antibodies can form immune complexes with allergens. These allergen-IgG immune complexes induce a regulatory immune response resulting in oral tolerance to the allergen. IgG also has suppressive effects on IgE-mediated activation of mast cells and basophils via IgG-mediated blocking and/or receptor-mediated inhibition. In addition, bovine IgG binds to a wide range of human bacteria and virusus, such as RSV, which could contribute to the allergy-protective capacity of raw cow's milk consumption. Lactoferrin (LF) protects against microbial pathogens by destabilizing the bacterial cell wall and by scavenging free iron in the intestine. The latter modulates the gut microbiome by inhibiting the growth of iron-dependent pathogens and by promoting the growth of bacteria with low iron requirements, such as *Bifidobacteria* and *Lactobacilli*. The presence of *Bifidobacteria*, *Lactobacilli*, and their metabolites (SCFA) in the gut seems to protect against allergic diseases. Lactoferrin also stimulates the production of TGF-β and IL-10 in the gut. These regulatory cytokines are also present in raw cow's milk and induce an environment favoring tolerance towards allergens. TGF-β enhances intestinal epithelial barrier function, induces different subclasses of regulatory T cells (FoxP3$^+$ regulatory T cells, Tr1 cells, Th3 cells), and favors IgA class switching. IL-10 inhibits antigen-presenting cell and eosinophil function and mast cell and Th2 cell activation. IL-10 furthermore induces IgG class switching and IL-10-producing Tr1 cells. The allergy-protective capacities of alkaline phosphatase (ALP) and osteopontin (OPN) are less clear. Alkaline phosphatase mainly reduces inflammatory responses by detoxifying bacterial LPS, which could prevent gut permeability and thereby migth impact food allergies. Osteopontin modulates both Th1 and Th2 immune responses; whether the net effect is protection against allergic diseases is currently unknown. Osteopontin furthermore acts as a carrier protein for lactoferrin. Together, the illustrated bioactive whey proteins might create an environment favoring unresponsiveness upon allergen exposure. Heating clearly affects the concentration as well as the functionality of these whey proteins (except for osteopontin), which might be detrimental to the allergy-protective effects of raw cow's milk consumption.

REFERENCES

1. European Academy of Allergy and Clinical Immunology. Global atlas of allergy. (2014) Available from: http://www.eaaci.org/GlobalAtlas/GlobalAtlasAllergy.pdf Accessed on: 18 October 2018
2. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, and Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2)LEN review. *Allergy* (2014) 69(10): 1275-9.
3. Strachan DP. Hay fever, hygiene, and household size. *BMJ* (1989) 299(6710): 1259-60.
4. Holgate ST and Polosa R. Treatment strategies for allergy and asthma. *Nat Rev Immunol* (2008) 8(3): 218-30.
5. von Mutius E and Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* (2010) 10(12): 861-8.
6. von Ehrenstein OS, von Mutius E, Illi S, Baumann L, Bohm O, and von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* (2000) 30(2): 187-93.
7. Braun-Fahrlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, *et al*. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. *Clin Exp Allergy* (1999) 29(1): 28-34.
8. Riedler J, Eder W, Oberfeld G, and Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin Exp Allergy* (2000) 30(2): 194-200.
9. Alfven T, Braun-Fahrlander C, Brunekreef B, von Mutius E, Riedler J, Scheynius A, *et al*. Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle - The PARSIFAL study. *Allergy* (2006) 61(4): 414-21.
10. Kilpelainen M, Terho EO, Helenius H, and Koskenvuo M. Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* (2000) 30(2): 201-8.
11. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
12. Remes ST, Iivanainen K, Koskela H, and Pekkanen J. Which factors explain the lower prevalence of atopy amongst farmers' children? *Clin Exp Allergy* (2003) 33(4): 427-34.
13. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
14. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, *et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
15. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
16. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T, *et al*. Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model. *Front Immunol* (2017) 8: 1045.
17. Oliver SP, Boor KJ, Murphy SC, and Murinda SE. Food safety hazards associated with consumption of raw milk. *Foodborne Pathog Dis* (2009) 6(7): 793-806.

18. Claeys WL, Cardoen S, Daube G, De Block J, Dewettinck K, Dierick K, *et al*. Raw or heated cow milk consumption: review of risks and benefits. *Food Control* (2013) 31(1): 251-62.
19. Committee on Infectious Diseases & Committee on Nutrition & American Academy of Pediatrics. Consumption of raw or unpasteurized milk and milk products by pregnant women and children. *Pediatrics* (2014) 133(1): 175-9.
20. Baars T. Milk consumption, raw and general, in the discussion on health or hazard. *J Nutr Ecol Food Res* (2013) 1(2): 91-107.
21. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
22. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-706 e13.
23. Chandan RC. Dairy ingredients for food processing: an overview in *Dairy Ingredients for Food Processing*. (2011): 3-33.
24. Verhoeckx KC, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S, *et al*. Food processing and allergenicity. *Food Chem Toxicol* (2015) 80: 223-40.
25. Rankin SA, Christiansen A, Lee W, Banavara DS, and Lopez-Hernandez A. Invited review: the application of alkaline phosphatase assays for the validation of milk product pasteurization. *J Dairy Sci* (2010) 93(12): 5538-51.
26. Michalski MC and Januel C. Does homogenization affect the human health properties of cow's milk? *Trends Food Sci Tech* (2006) 17(8): 423-37.
27. Perkin MR. Unpasteurized milk: health or hazard? *Clin Exp Allergy* (2007) 37(5): 627-30.
28. Poulsen OM, Hau J, and Kollerup J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. *Clin Allergy* (1987) 17(5): 449-58.
29. Poulsen OM, Nielsen BR, Basse A, and Hau J. Comparison of intestinal anaphylactic reactions in sensitized mice challenged with untreated bovine milk and homogenized bovine milk. *Allergy* (1990) 45(5): 321-6.
30. Pelto L, Rantakokko HK, Lilius EM, Nuutila J, and Salminen S. No difference in symptoms and receptor expression in lactose-intolerant and in milk-hypersensitive subjects following intake of homogenized and unhomogenized milk. *Int Dairy J* (2000) 10(11): 799-803.
31. Host A and Samuelsson EG. Allergic reactions to raw, pasteurized, and homogenized/pasteurized cow milk: a comparison. A double-blind placebo-controlled study in milk allergic children. *Allergy* (1988) 43(2): 113-8.
32. Michalski MC. On the supposed influence of milk homogenization on the risk of CVD, diabetes and allergy. *Br J Nutr* (2007) 97(4): 598-610.
33. Guetouache M, Guessas B, and Medjekal S. Composition and nutritional value of raw milk. *Issues Biol Sci Pharm Res* (2014) 2: 115-22.
34. Braun-Fahrlander C and von Mutius E. Can farm milk consumption prevent allergic diseases? *Clin Exp Allergy* (2011) 41(1): 29-35.
35. Schrezenmeir J, Korhonen H, Williams C, Gill HS, and Shah N. Foreword. *Brit J Nutr* (2000) 84(S1): 1.
36. Perdijk O, van Splunter M, Savelkoul HFJ, Brugman S, and van Neerven RJJ. Cow's milk and immune function in the respiratory tract: potential mechanisms. *Front Immunol* (2018) 9: 143.
37. Mehra R, Marnila P, and Korhonen H. Milk immunoglobulins for health promotion. *Int Dairy J* (2006) 16(11): 1262-71.
38. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
39. Mosconi E, Rekima A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, *et al*. Breast milk immune complexes are potent inducers

of oral tolerance in neonates and prevent asthma development. *Mucosal Immunol* (2010) 3(5): 461-74.
40. Collins AM, Roberton DM, Hosking CS, and Flannery GR. Bovine milk, including pasteurised milk, contains antibodies directed against allergens of clinical importance to man. *Int Arch Allergy Appl Immunol* (1991) 96(4): 362-7.
41. Ober RJ, Radu CG, Ghetie V, and Ward ES. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. *Int Immunol* (2001) 13(12): 1551-9.
42. Li-Chan E, Kummer A, Losso JN, and Nakai S. Survey of immunoglobulin G content and antibody specificity in cows' milk from British Columbia. *Food Agr Immunol* (1994) 6(4): 443-51.
43. Yolken RH, Losonsky GA, Vonderfecht S, Leister F, and Wee SB. Antibody to human rotavirus in cow's milk. *N Engl J Med* (1985) 312(10): 605-10.
44. Korhonen H, Marnila P, and Gill HS. Bovine milk antibodies for health. *Br J Nutr* (2007) 84(S1): 135-46.
45. den Hartog G, Jacobino S, Bont L, Cox L, Ulfman LH, Leusen JHW, *et al*. Specificity and effector functions of human RSV-specific IgG from bovine milk. *PLoS One* (2014) 9(11): e112047.
46. Pérez-Yarza EG, Moreno A, Lázaro P, Mejías A, and Ramilo O. The association between respiratory syncytial virus infection and the development of childhood asthma: a systematic review of the literature. *Pediatr Infect Dis J* (2007) 26(8): 733-9.
47. Régnier SA and Huels J. Association between respiratory syncytial virus hospitalizations in infants and respiratory sequelae: systematic review and meta-analysis. *Pediatr Infect Dis J* (2013) 32(8): 820-6.
48. Malbec O and Daëron M. The mast cell IgG receptors and their roles in tissue inflammation. *Immunol Rev* (2007) 217: 206-21.
49. Renz H, Allen KJ, Sicherer SH, Sampson HA, Lack G, Beyer K, *et al*. Food allergy. *Nat Rev Dis Primers* (2018) 4: 17098.
50. Burton OT, Tamayo JM, Stranks AJ, Koleoglou KJ, and Oettgen HC. Allergen-specific IgG antibody signaling through FcgammaRIIb promotes food tolerance. *J Allergy Clin Immunol* (2018) 141(1): 189-201 e3.
51. Burton OT, Logsdon SL, Zhou JS, Medina-Tamayo J, Abdel-Gadir A, Noval Rivas M, *et al*. Oral immunotherapy induces IgG antibodies that act through FcgammaRIIb to suppress IgE-mediated hypersensitivity. *J Allergy Clin Immunol* (2014) 134(6): 1310-7 e6.
52. Daëron M, Malbec O, Latour S, Arock M, and Fridman WH. Regulation of high-affinity IgE receptor-mediated mast cell activation by murine low-affinity IgG receptors. *Journal Clin Invest* (1995) 95(2): 577-85.
53. Cacho NT and Lawrence RM. Innate immunity and breast milk. *Front Immunol* (2017) 8: 584.
54. Giansanti F, Panella G, Leboffe L, and Antonini G. Lactoferrin from milk: nutraceutical and pharmacological properties. *Pharmaceuticals* (2016) 9(4): 61.
55. Munblit D, Peroni DG, Boix-Amoros A, Hsu PS, Van't Land B, Gay MCL, *et al*. Human milk and allergic diseases: an unsolved puzzle. *Nutrients* (2017) 9(8): 894.
56. Legrand D. Overview of lactoferrin as a natural immune modulator. *J Pediatr* (2016) 173: S10-5.
57. Legrand D. Lactoferrin, a key molecule in immune and inflammatory processes. *Biochem Cell Biol* (2012) 90(3): 252-68.
58. Fischer R, Debbabi H, Dubarry M, Boyaka P, and Tome D. Regulation of physiological and pathological Th1 and Th2 responses by lactoferrin. *Biochem Cell Biol* (2006) 84(3): 303-11.
59. Oda H, Wakabayashi H, Yamauchi K, and Abe F. Lactoferrin and bifidobacteria. *Biometals* (2014) 27(5): 915-22.
60. Tian H, Maddox IS, Ferguson LR, and Shu Q. Influence of bovine lactoferrin on selected probiotic bacteria and intestinal pathogens.

Biometals (2010) 23(3): 593-6.

61. Kalliomäki M, Kirjavainen P, Eerola E, Kero P, Salminen S, and Isolauri E. Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* (2001) 107(1): 129-34.
62. Björkstén B, Sepp E, Julge K, Voor T, and Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* (2001) 108(4): 516-20.
63. Sjögren YM, Jenmalm MC, Böttcher MF, Björkstén B, and Sverremark-Ekström E. Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clin Exp Allergy* (2009) 39(4): 518-26.
64. McKenzie C, Tan J, Macia L, and Mackay CR. The nutrition-gut microbiome-physiology axis and allergic diseases. *Immunol Rev* (2017) 278(1): 277-95.
65. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, and Macia L. Chapter three - The role of short-chain fatty acids in health and disease in *Advances in Immunology*, F.W. Alt, Editor. (2014); 91-119.
66. Wang CC, Wu H, Lin FH, Gong R, Xie F, Peng Y*, et al*. Sodium butyrate enhances intestinal integrity, inhibits mast cell activation, inflammatory mediator production and JNK signaling pathway in weaned pigs. *Innate Immun* (2018) 24(1): 40-6.
67. Kruzel ML, Bacsi A, Choudhury B, Sur S, and Boldogh I. Lactoferrin decreases pollen antigen-induced allergic airway inflammation in a murine model of asthma. *Immunology* (2006) 119(2): 159-66.
68. Liao Y, Jiang R, and Lonnerdal B. Biochemical and molecular impacts of lactoferrin on small intestinal growth and development during early life. *Biochem Cell Biol* (2012) 90(3): 476-84.
69. Shin K, Wakabayashi H, Yamauchi K, Yaeshima T, and Iwatsuki K. Recombinant human intelectin binds bovine lactoferrin and its peptides. *Biol Pharm Bull* (2008) 31(8): 1605-8.
70. Lonnerdal B, Jiang R, and Du X. Bovine lactoferrin can be taken up by the human intestinal lactoferrin receptor and exert bioactivities. *J Pediatr Gastroenterol Nutr* (2011) 53(6): 606-14.
71. Puddu P, Latorre D, Carollo M, Catizone A, Ricci G, Valenti P*, et al*. Bovine lactoferrin counteracts Toll-like receptor mediated activation signals in antigen presenting cells. *PLoS One* (2011) 6(7): e22504.
72. Chen K, Chai L, Li H, Zhang Y, Xie HM, Shang J*, et al*. Effect of bovine lactoferrin from iron-fortified formulas on diarrhea and respiratory tract infections of weaned infants in a randomized controlled trial. *Nutrition* (2016) 32(2): 222-7.
73. Chatterton DE, Nguyen DN, Bering SB, and Sangild PT. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int J Biochem Cell Biol* (2013) 45(8): 1730-47.
74. Rautava S and Walker WA. Academy of Breastfeeding Medicine Founder's Lecture 2008: Breastfeeding - An extrauterine link between mother and child. *Breastfeed Med* (2009) 4(1): 3-10.
75. Oddy WH and Rosales F. A systematic review of the importance of milk TGF-beta on immunological outcomes in the infant and young child. *Pediatr Allergy Immunol* (2010) 21(1 Pt 1): 47-59.
76. Howe KL, Reardon C, Wang A, Nazli A, and McKay DM. Transforming growth factor-beta regulation of epithelial tight junction proteins enhances barrier function and blocks enterohemorrhagic Escherichia coli O157:H7-induced increased permeability. *Am J Pathol* (2005) 167(6): 1587-97.
77. Planchon SM, Martins CA, Guerrant RL, and Roche JK. Regulation of intestinal epithelial barrier function by TGF-beta 1. Evidence for its role in abrogating the effect of a T cell cytokine. *J Immunol* (1994) 153(12): 5730-9.
78. Roche JK, Martins CA, Cosme R, Fayer R, and Guerrant RL. Transforming growth factor beta1 ameliorates intestinal epithelial barrier

disruption by Cryptosporidium parvum in vitro in the absence of mucosal T lymphocytes. *Infect Immun* (2000) 68(10): 5635-44.
79. Kotler BM, Kerstetter JE, and Insogna KL. Claudins, dietary milk proteins, and intestinal barrier regulation. *Nutr Rev* (2013) 71(1): 60-5.
80. Groschwitz KR and Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* (2009) 124(1): 3-20.
81. Scott CL, Aumeunier AM, and Mowat AM. Intestinal $CD103^+$ dendritic cells: master regulators of tolerance? *Trends Immunol* (2011) 32(9): 412-9.
82. Verhasselt V, Milcent V, Cazareth J, Kanda A, Fleury S, Dombrowicz D*, et al*. Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nat Med* (2008) 14(2): 170-5.
83. Penttila IA, Flesch IE, McCue AL, Powell BC, Zhou FH, Read LC*, et al*. Maternal milk regulation of cell infiltration and interleukin 18 in the intestine of suckling rat pups. *Gut* (2003) 52(11): 1579-86.
84. Penttila I. Effects of transforming growth factor-beta and formula feeding on systemic immune responses to dietary beta-lactoglobulin in allergy-prone rats. *Pediatr Res* (2006) 59(5): 650-5.
85. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N*, et al*. Conversion of Peripheral $CD4^+CD25^-$ naive T cells to $CD4^+CD25^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J Exp Med* (2003) 198(12): 1875-86.
86. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, and von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* (2005) 6(12): 1219-27.
87. Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY*, et al*. Regulatory T cells expressing interleukin 10 develop from $Foxp3^+$ and $Foxp3^-$ precursor cells in the absence of interleukin 10. *Nat Immunol* (2007) 8(9): 931-41.
88. Faria AM and Weiner HL. Oral tolerance. *Immunol Rev* (2005) 206: 232-59.
89. Perez-Machado MA, Ashwood P, Thomson MA, Latcham F, Sim R, Walker-Smith JA*, et al*. Reduced transforming growth factor-beta1-producing T cells in the duodenal mucosa of children with food allergy. *Eur J Immunol* (2003) 33(8): 2307-15.
90. Janzi M, Kull I, Sjöberg R, Wan J, Melén E, Bayat N*, et al*. Selective IgA deficiency in early life: association to infections and allergic diseases during childhood. *Clin Immunol* (2009) 133(1): 78-85.
91. Savilahti E, Siltanen M, Kajosaari M, Vaarala O, and Saarinen KM. IgA antibodies, TGF-beta1 and -beta2, and soluble CD14 in the colostrum and development of atopy by age 4. *Pediatr Res* (2005) 58(6): 1300-5.
92. Jarvinen KM, Laine ST, Jarvenpaa AL, and Suomalainen HK. Does low IgA in human milk predispose the infant to development of cow's milk allergy? *Pediatr Res* (2000) 48(4): 457-62.
93. Hawrylowicz CM and O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* (2005) 5(4): 271-83.
94. Royer B, Varadaradjalou S, Saas P, Guillosson JJ, Kantelip JP, and Arock M. Inhibition of IgE-induced activation of human mast cells by IL-10. *Clin Exp Allergy* (2001) 31(5): 694-704.
95. Takanaski S, Nonaka R, Xing Z, O'Byrne P, Dolovich J, and Jordana M. Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *J Exp Med* (1994) 180(2): 711-5.
96. Grunig G, Corry DB, Leach MW, Seymour BW, Kurup VP, and Rennick DM. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. *J Exp Med* (1997) 185(6): 1089-99.
97. de Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, te Velde A, Figdor C*, et al*. Interleukin 10 (IL-10) and viral IL-10

strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med* (1991) 174(4): 915-24.

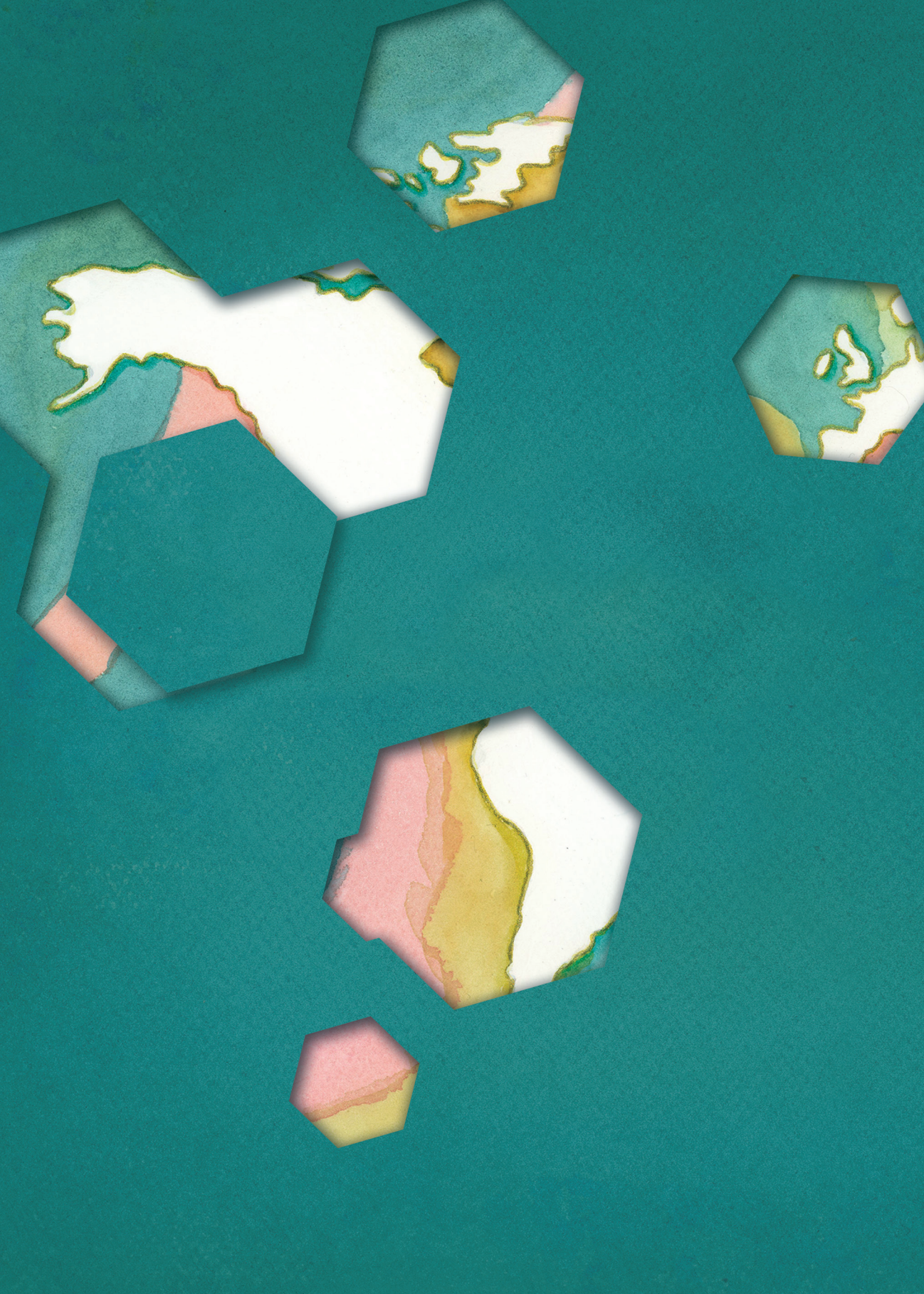
98. Buelens C, Verhasselt V, De Groote D, Thielemans K, Goldman M, and Willems F. Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor. *Eur J Immunol* (1997) 27(3): 756-62.
99. Jeannin P, Lecoanet S, Delneste Y, Gauchat JF, and Bonnefoy JY. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol* (1998) 160(7): 3555-61.
100. Levings MK, Gregori S, Tresoldi E, Cazzaniga S, Bonini C, and Roncarolo MG. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not $CD25^{+}CD4^{+}$ Tr cells. *Blood* (2005) 105(3): 1162-9.
101. Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J, and Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. *J Allergy Clin Immunol* (1996) 97(6): 1288-96.
102. Heaton T, Rowe J, Turner S, Aalberse RC, de Klerk N, Suriyaarachchi D*, et al*. An immunoepidemiological approach to asthma: identification of in-vitro T cell response patterns associated with different wheezing phenotypes in children. *The Lancet* (2005) 365(9454): 142-9.
103. den Hartog G, Savelkoul HF, Schoemaker R, Tijhaar E, Westphal AH, de Ruiter T*, et al*. Modulation of human immune responses by bovine interleukin-10. *PLoS One* (2011) 6(3): e18188.
104. Pletinckx K, Dohler A, Pavlovic V, and Lutz MB. Role of dendritic cell maturity/costimulation for generation, homeostasis, and suppressive activity of regulatory T cells. *Front Immunol* (2011) 2: 39.
105. Takakura N, Wakabayashi H, Yamauchi K, and Takase M. Influences of orally administered lactoferrin on IFN-gamma and IL-10 production by intestinal intraepithelial lymphocytes and mesenteric lymph-node cells. *Biochem Cell Biol* (2006) 84(3): 363-8.
106. Lalles JP. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. *Nutr Rev* (2010) 68(6): 323-32.
107. Lalles JP. Dairy products and the French paradox: could alkaline phosphatases play a role? *Med Hypotheses* (2016) 92: 7-11.
108. Morton RK. Alkaline phosphatase of milk. 1. Association of the enzyme with a particulate lipoprotein complex. *Biochem J* (1953) 55(5): 786-95.
109. Beumer C, Wulferink M, Raaben W, Fiechter D, Brands R, and Seinen W. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. *J Pharmacol Exp Ther* (2003) 307(2): 737-44.
110. Whitehouse JS, Riggle KM, Purpi DP, Mayer AN, Pritchard KA, Jr., Oldham KT*, et al*. The protective role of intestinal alkaline phosphatase in necrotizing enterocolitis. *J Surg Res* (2010) 163(1): 79-85.
111. Tuin A, Poelstra K, de Jager-Krikken A, Bok L, Raaben W, Velders MP*, et al*. Role of alkaline phosphatase in colitis in man and rats. *Gut* (2009) 58(3): 379-87.
112. Bender B, Baranyi M, Kerekes A, Bodrogi L, Brands R, Uhrin P*, et al*. Recombinant human tissue non-specific alkaline phosphatase successfully counteracts lipopolysaccharide induced sepsis in mice. *Physiol Res* (2015) 64(5): 731-8.
113. Iyngkaran N, Yadav M, and Boey CG. Mucosal and serum alkaline phosphatase activities in milk allergy. *Med J Malaysia* (1995) 50(1): 21-4.
114. Demmelmair H, Prell C, Timby N, and Lonnerdal B. Benefits of lactoferrin, osteopontin and milk fat globule membranes for infants. *Nutrients* (2017) 9(8): 817.

115. Frenzel DF and Weiss JM. Osteopontin and allergic disease: pathophysiology and implications for diagnostics and therapy. *Expert Rev Clin Immunol* (2011) 7(1): 93-109.
116. Renkl AC, Wussler J, Ahrens T, Thoma K, Kon S, Uede T, *et al*. Osteopontin functionally activates dendritic cells and induces their differentiation toward a Th1-polarizing phenotype. *Blood* (2005) 106(3): 946-55.
117. Nau GJ, Liaw L, Chupp GL, Berman JS, Hogan BL, and Young RA. Attenuated host resistance against Mycobacterium bovis BCG infection in mice lacking osteopontin. *Infect Immun* (1999) 67(8): 4223-30.
118. Koguchi Y, Kawakami K, Uezu K, Fukushima K, Kon S, Maeda M, *et al*. High plasma osteopontin level and its relationship with interleukin-12-mediated type 1 T helper cell response in tuberculosis. *Am J Respir Crit Care Med* (2003) 167(10): 1355-9.
119. Sato T, Nakai T, Tamura N, Okamoto S, Matsuoka K, Sakuraba A, *et al*. Osteopontin/Eta-1 upregulated in Crohn's disease regulates the Th1 immune response. *Gut* (2005) 54(9): 1254-62.
120. Comabella M, Pericot I, Goertsches R, Nos C, Castillo M, Blas Navarro J, *et al*. Plasma osteopontin levels in multiple sclerosis. *J Neuroimmunol* (2005) 158(1-2): 231-9.
121. Xu G, Nie H, Li N, Zheng W, Zhang D, Feng G, *et al*. Role of osteopontin in amplification and perpetuation of rheumatoid synovitis. *J Clin Invest* (2005) 115(4): 1060-7.
122. Konno S, Golden DB, Schroeder J, Hamilton RG, Lichtenstein LM, and Huang SK. Increased expression of osteopontin is associated with long-term bee venom immunotherapy. *J Allergy Clin Immunol* (2005) 115(5): 1063-7.
123. Kurokawa M, Konno S, Takahashi A, Plunkett B, Rittling SR, Matsui Y, *et al*. Regulatory role of DC-derived osteopontin in systemic allergen sensitization. *Eur J Immunol* (2009) 39(12): 3323-30.
124. Nagasaka A, Matsue H, Matsushima H, Aoki R, Nakamura Y, Kambe N, *et al*. Osteopontin is produced by mast cells and affects IgE-mediated degranulation and migration of mast cells. *Eur J Immunol* (2008) 38(2): 489-99.
125. Xanthou G, Alissafi T, Semitekolou M, Simoes DC, Economidou E, Gaga M, *et al*. Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets. *Nat Med* (2007) 13(5): 570-8.
126. Samitas K, Zervas E, Vittorakis S, Semitekolou M, Alissafi T, Bossios A, *et al*. Osteopontin expression and relation to disease severity in human asthma. *Eur Respir J* (2011) 37(2): 331-41.
127. Jiang R and Lonnerdal B. Biological roles of milk osteopontin. *Curr Opin Clin Nutr Metab Care* (2016) 19(3): 214-9.
128. Yamniuk AP, Burling H, and Vogel HJ. Thermodynamic characterization of the interactions between the immunoregulatory proteins osteopontin and lactoferrin. *Mol Immunol* (2009) 46(11-12): 2395-402.
129. Desmasures N, Bazin F, and Gueguen M. Microbiological composition of raw milk from selected farms in the Camembert region of Normandy. *J Appl Microbiol* (1997) 83(1): 53-8.
130. Suhren B, Hesselbarth H, Heeschen W, and Südi J. Evaluation of the lipopolysaccharide (LPS) content as determined by the Limulus test in milk and milk products. II. Raw milk and influences of technological procedures. *Milchwissenschaft* (1986) 41(3): 156-60.
131. Sipka S, Beres A, Bertok L, Varga T, and Bruckner G. Comparison of endotoxin levels in cow's milk samples derived from farms and shops. *Innate Immun* (2015) 21(5): 531-6.
132. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, *et al*. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* (2002) 347(12): 869-77.
133. Gehring U, Spithoven J, Schmid S, Bitter S, Braun-Fahrlander C, Dalphin JC, *et al*. Endotoxin levels in cow's milk samples from farming and non-farming families - The PASTURE study. *Environ Int* (2008) 34(8): 1132-6.

134. Melnik BC, John SM, and Schmitz G. Milk: an exosomal microRNA transmitter promoting thymic regulatory T cell maturation preventing the development of atopy? *J Transl Med* (2014) 12(1): 43.
135. Annes JP, Munger JS, and Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci* (2003) 116(Pt 2): 217-24.
136. Ando T, Hatsushika K, Wako M, Ohba T, Koyama K, Ohnuma Y, *et al*. Orally administered TGF-beta is biologically active in the intestinal mucosa and enhances oral tolerance. *J Allergy Clin Immunol* (2007) 120(4): 916-23.
137. Slavin AJ, Maron R, and Weiner HL. Mucosal administration of IL-10 enhances oral tolerance in autoimmune encephalomyelitis and diabetes. *Int Immunol* (2001) 13(6): 825-33.
138. Troost FJ, Steijns J, Saris WH, and Brummer RJ. Gastric digestion of bovine lactoferrin in vivo in adults. *J Nutr* (2001) 131(8): 2101-4.
139. Davidson LA and Lonnerdal B. Persistence of human milk proteins in the breast-fed infant. *Acta Paediatr Scand* (1987) 76(5): 733-40.
140. Dallas DC, Underwood MA, Zivkovic AM, and German JB. Digestion of protein in premature and term infants. *J Nutr Disord Ther* (2012) 2(3): 112.
141. Britton JR and Koldovsky O. Luminal digestion of lactoferrin in suckling and weanling rats. *Am J Physiol* (1987) 253(3 Pt 1): G397-403.
142. Jasion VS and Burnett BP. Survival and digestibility of orally-administered immunoglobulin preparations containing IgG through the gastrointestinal tract in humans. *Nutr J* (2015) 14: 22.
143. Ulfman LH, Leusen JHW, Savelkoul HFJ, Warner JO, and van Neerven RJJ. Effects of bovine immunoglobulins on immune function, allergy, and infection. *Front Nutr* (2018) 5: 52.
144. Payne RB. The controlling effect of carbohydrate in human, rabbit and bovine immunoglobulin G on proteolysis by papin. *Biochem J* (1969) 111(4): 473-8.
145. Lalles JP. Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr Rev* (2014) 72(2): 82-94.
146. Bol-Schoenmakers M, Fiechter D, Raaben W, Hassing I, Bleumink R, Kruijswijk D, *et al*. Intestinal alkaline phosphatase contributes to the reduction of severe intestinal epithelial damage. *Eur J Pharmacol* (2010) 633(1-3): 71-7.
147. Martinez-Moya P, Ortega-Gonzalez M, Gonzalez R, Anzola A, Ocon B, Hernandez-Chirlaque C, *et al*. Exogenous alkaline phosphatase treatment complements endogenous enzyme protection in colonic inflammation and reduces bacterial translocation in rats. *Pharmacol Res* (2012) 66(2): 144-53.
148. Brick T, Ege M, Boeren S, Bock A, von Mutius E, Vervoort J, *et al*. Effect of processing intensity on immunologically active bovine milk serum proteins. *Nutrients* (2017) 9(9): 963.
149. Mainer G, Sanchez L, Ena JM, and Calvo M. Kinetic and thermodynamic parameters for heat denaturation of bovine milk IgG, IgA and IgM. *J Food Sci* (1997) 62(5): 1034-8.
150. Korhonen H, Marnila P, and Gill HS. Milk immunoglobulins and complement factors. *Br J Nutr* (2000) 84: S75-80.
151. Li-Chan E, Kummer A, Losso JN, Kitts DD, and Nakai S. Stability of bovine immunoglobulins to thermal-treatment and processing. *Food Res Int* (1995) 28(1): 9-16.
152. Adhisivam B, Vishnu Bhat B, Rao K, Kingsley SM, Plakkal N, and Palanivel C. Effect of Holder pasteurization on macronutrients and immunoglobulin profile of pooled donor human milk. *J Matern Fetal Neonatal Med* (2018): 1-4.
153. Schwarcz WD, Carnelocce L, Silva JL, Oliveira AC, and Goncalves RB. Conformational changes in bovine lactoferrin induced by slow or fast temperature increases. *Biol Chem* (2008) 389(8): 1137-42.
154. Zhang LN, Boeren S, Smits M, van Hooijdonk T, Vervoort J, and Hettinga K. Proteomic study

on the stability of proteins in bovine, camel, and caprine milk sera after processing. *Food Res Int* (2016) 82: 104-11.

155. Lorenzen PC, Clawin-Raedecker I, Einhoff K, Hammer P, Hartmann R, Hoffmann W*, et al*. A survey of the quality of extended shelf life (ESL) milk in relation to HTST and UHT milk. *Int J Dairy Technol* (2011) 64(2): 166-78.
156. Peroni DG, Piacentini GL, Bodini A, Pigozzi R, and Boner AL. Transforming growth factor-beta is elevated in unpasteurized cow's milk. *Pediatr Allergy Immunol* (2009) 20(1): 42-4.
157. Elfstrand L, Lindmark-Mansson H, Paulsson M, Nyberg L, and Akesson B. Immunoglobulins, growth factors and growth hormone in bovine colostrum and the effects of processing. *Int Dairy J* (2002) 12(11): 879-87.
158. Hodgkinson AJ, McDonald NA, and Hine B. Effect of raw milk on allergic responses in a murine model of gastrointestinal allergy. *Br J Nutr* (2014) 112(3): 390-7.
159. Akbache A, Rocafi A, Saffon M, Lamiot E, Moroni O, Turgeon S*, et al*. Effect of heating on the distribution of transforming growth factor-beta 2 in bovine milk. *Food Res Int* (2011) 44(1): 28-32.
160. Untalan PB, Keeney SE, Palkowetz KH, Rivera A, and Goldman AS. Heat susceptibility of interleukin-10 and other cytokines in donor human milk. *Breastfeed Med* (2009) 4(3): 137-44.
161. Ewaschuk JB, Unger S, O'Connor DL, Stone D, Harvey S, Clandinin MT*, et al*. Effect of pasteurization on selected immune components of donated human breast milk. *J Perinatol* (2011) 31(9): 593-8.
162. Wijayanti HB, Bansal N, and Deeth HC. Stability of whey proteins during thermal processing: a review. *Compr Rev Food Sci F* (2014) 13(6): 1235-51.



CHAPTER 3

Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model

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ABSTRACT

Epidemiological studies show an inverse relation between raw cow's milk consumption and the development of asthma. This protective effect seems to be abolished by milk processing. However, evidence for a causal relationship is lacking, and direct comparisons between raw and processed milk are hardly studied. Therefore, this study investigated the preventive capacity of raw and heated raw milk on the development of house dust mite (HDM)-induced allergic asthma in mice. Six- to seven-week-old male BALB/c mice were intranasally (i.n.) sensitized with 1 µg HDM or PBS on day 0, followed by an i.n. challenge with 10 µg HDM or PBS on days 7-11. In addition, mice were fed 0.5 mL raw cow's milk, heated raw cow's milk, or PBS three times a week throughout the study, starting 1 day before sensitization. On day 14, airway hyperresponsiveness (AHR) in response to increasing doses of methacholine was measured to assess lung function. Bronchoalveolar lavage fluid and lungs were furthermore collected to study the extent of airway inflammation. Raw milk prevented both HDM-induced AHR and pulmonary eosinophilic inflammation, whereas heated raw milk did not. Both milk types suppressed the Th2-polarizing chemokine CCL17 in lung homogenates and reduced lung Th2 and Th17 cell frequency. IL-4 and IL-13 production after *ex vivo* restimulation of lung T cells with HDM was also reduced by both milk types. However, local IL-5 and IL-13 concentrations were only suppressed by raw milk. These findings support the asthma-protective capacity of raw cow's milk and show the importance of reduced local type 2 cytokine levels. Heated raw milk did not show an asthma-protective effect, which indicates the involvement of heat-sensitive components. Besides causal evidence, this study provides the basis for further mechanistic studies.

INTRODUCTION

With up to 300 million people affected worldwide, asthma is a global burden (1). It is one of the most common chronic diseases in the world, and the prevalence has increased significantly in Western countries in the last decades (2). The disease is characterized by airway hyperresponsiveness (AHR), eosinophilic airway inflammation, and excessive mucus secretion. This leads to shortness of breath, wheezing, coughing, and chest tightness in genetically susceptible individuals. Asthma can be triggered by a wide range of environmental stimuli, such as inhaled allergens [e.g., house dust mite (HDM), animal dander], respiratory infections, and airborne pollutants (e.g., tobacco smoke, air pollution) (3).

The remarkable increase in the prevalence of asthma and allergies in Western countries in recent decades is often explained by the loss of rural living conditions and associated changes in diet and lifestyle (4). This so-called 'hygiene hypothesis' is supported by numerous epidemiological studies that consistently show that growing up on a farm reduces the risk of developing asthma and allergies (5-8). Farm-associated exposures contributing to this protective effect are contact with livestock, contact with animal feed, and the consumption of raw, unprocessed, cow's milk (9-13). The protective effect of raw cow's milk consumption is of particular interest since this was found to be independent of concomitant farm exposures and farm status (10, 12, 13). The latter suggests that non-farming populations might also benefit from the protective effects of raw cow's milk consumption.

Interestingly, cross-sectional evidence suggests that the asthma- and allergy-protective effects of raw cow's milk are abolished by milk processing (9). Processed cow's milk used for commercial purposes differs in many respects from raw cow's milk. Commercial milk is usually homogenized and heat-treated to increase shelf life and to inactivate potentially pathogenic microorganisms (14). However, this milk processing also affects other milk components. The structure of the milk fat, for example, is changed remarkably by homogenization, and this might alter allergen presentation (15). In addition, heat-sensitive milk components, such as proteins, can be structurally altered upon heating and consequently lose functionality (16). These changes might (partly) explain why the beneficial farm milk effect seems to be lost after processing (9).

At the moment, raw milk can theoretically be considered as preventive treatment, but in reality, its consumption is limited due to its possible contamination with pathogens. To produce safe and protective milk, components and mechanisms underlying the asthma-protective effect need to be elucidated. At the same time, the current epidemiological evidence needs to be confirmed by causality. In the present study, we therefore examined the ability of raw cow's

milk consumption to prevent the development of asthma in a murine HDM-induced allergic asthma model. Because many differences between raw milk and commercial milk make it difficult to disentangle the different effects of milk processing, we compared raw and heated raw cow's milk to solely assess the effects of heating.

MATERIALS & METHODS

Mice

Six- to seven-week-old, specific pathogen-free, male BALB/c mice (*n* = 9/group) were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed at the animal facility of Utrecht University (Utrecht, The Netherlands) under bio-contained sterile conditions using HEPA® filtered isocages® (Tecniplast, Buguggiate, Italy). Food and water were provided *ad libitum*. All animal procedures were conducted in accordance with the governmental guidelines and approved by the Ethical Committee for Animal Research of the Utrecht University (DEC2014.III.12.117).

HDM-induced asthma model

A schematic overview of the experimental design is shown in **Figure 1**. Anesthetized mice were intranasally (i.n.) sensitized with PBS or 1 µg HDM/40 µL PBS (Greer Laboratories Inc., Lenoir, NC, USA) on day 0 followed by an i.n. challenge with PBS or 10 µg HDM/40 µL PBS on days 7-11 (17). Three times a week throughout the study, starting one day before sensitization, mice were fed 0.5 mL raw cow's milk, heated raw cow's milk, or PBS by oral gavage. Raw cow's milk was collected from a biodynamic dairy farm (Hof Dannwisch, Horst, Germany). One day after collection, part of the raw milk was aliquoted and stored at -20 °C until further use. The remainder was heated for 10 min at 80 °C in a water bath, allowed to cool to room temperature, aliquoted and then stored at -20 °C until further use. Mice were killed on day 14.

Airway responsiveness

Mice were intraperitoneally (i.p.) anesthetized with a mixture of ketamine (Vétoquinol SA, Lure, France; 125 mg/kg) and medetomidine (Pfizer Animal Health BV, Capelle aan den IJssel, The Netherlands; 0.4 mg/kg). EMKA invasive measurement of dynamic resistance (EMKA Technologies, Paris, France) in response to increasing doses of methacholine (acetyl-β-methyl-choline chloride; Sigma-Aldrich, Zwijndrecht, The Netherlands; 0-25 mg/mL, 10% puff for 10 s) was used on day 14 to assess lung function. Average lung resistance (R_L) is presented in cm H_2O/(mL/s) (18).

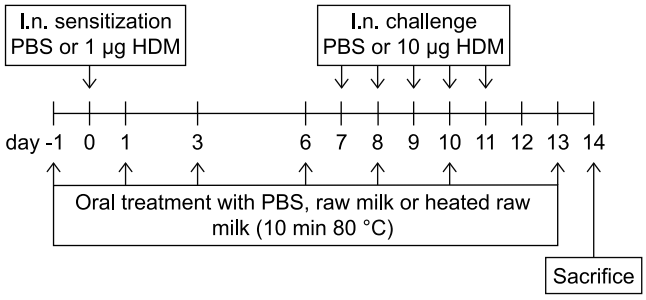


Figure 1. Schematic overview of the study design. Male BALB/c mice (*n* = 9/group) were sensitized intranasally (i.n.) with PBS or house dust mite (HDM) on day 0 and were challenged i.n. on days 7-11 with PBS or HDM. By oral gavage, the mice were given raw cow's milk, heated raw cow's milk or PBS three times a week throughout the study, starting one day before sensitization. Mice were killed on day 14.

Bronchoalveolar lavage

On day 14, mice were killed using an i.p. overdose of pentobarbital (Nembutal™; Ceva Santé Animale, Naaldwijk, The Netherlands; 600 mg/kg). Lungs were lavaged through a tracheal cannula with 1 mL of pyrogen-free saline (0.9% NaCl, 37 °C) supplemented with protease inhibitors (cOmplete, Mini Protease Inhibitor Cocktail; Roche Diagnostics, Mannheim, Germany). This was followed by three lavages with 1 mL saline solution (0.9% NaCl, 37 °C). The bronchoalveolar lavage fluid (BALF) was centrifuged (400 *g*, 5 min), and pellets of the four lavages were pooled. Total numbers of BALF cells were counted using a Bürker-Türk chamber (magnification 100×). For differential BALF cell counts, cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Dudingen, Switzerland). Numbers of eosinophils, neutrophils, lymphocytes, and macrophages were scored using light microscopy. At least 200 cells were counted, and the absolute number of each cell type was calculated (19).

Preparation of lung homogenates

Lung samples were homogenized in 1% Trition X-100 (Sigma-Aldrich)/PBS containing protease inhibitors (cOmplete, Mini Protease Inhibitor Cocktail; Roche Diagnostics) using a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Homogenates were centrifuged (14,000 rpm, 10 min), and supernatants were collected and stored at -20 °C until further use. Prior to cytokine and chemokine analysis, the protein concentration of each sample was determined by using the Pierce BCA protein assay kit standardized to bovine serum albumin (BSA) according to the manufacturer's protocol (Thermo Fisher Scientific, Paisley, Scotland). Homogenates were subsequently diluted to a final concentration of 1 mg protein/mL.

***Ex vivo* lung restimulation with HDM**

Single lung cell suspensions were obtained by cutting lung samples into small pieces and by adding digestion buffer containing DNase I and Collagenase A (Roche Diagnostics) for 30 min. The digestion was stopped by adding fetal bovine serum (FBS; Bodinco, Alkmaar, The

Netherlands). Lung pieces were passed through a 70 µm nylon cell strainer and rinsed with 10 mL RPMI. Cells were washed and resuspended in RPMI 1640 culture medium (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated FBS and 0.1% penicillin-streptomycin solution (Sigma-Aldrich). Lung cells (4×10^5 cells/well) were cultured in medium with or without 50 µg/mL HDM. Supernatant was harvested after four days of culture (37 °C, 5% CO_2) and stored at -20 °C until further analysis.

Measurement of cytokines and chemokines

IL-33, CCL20, CCL17, and CCL22 concentrations were measured in lung homogenates with a DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) and IL-5 and IL-13 with a Ready-SET-Go!® ELISA (eBioscience, Breda, The Netherlands). A standard IL-13 flex set or a standard Th1/Th2/Th17 assay (BD Biosciences, Alphen aan de Rijn, The Netherlands) was used to determine cytokine concentrations in supernatants of lung restimulation. All assays were performed according to the manufacturer's protocol. The concentrations of cytokines and chemokines were expressed as picogram per milligram protein in lung homogenates and picogram per milliliter in lung restimulation supernatants.

Flow cytometric analysis of immune cells in the lung

Single lung cell suspensions were resuspended in PBS/1% BSA (Sigma-Aldrich) and incubated for 15 min at 4 °C with Fc block CD16/CD32 antibodies (BD Biosciences; 5 µg/mL) to prevent non-antigen-specific binding. Cells were subsequently stained with antibodies (eBioscience, unless otherwise stated) against CD4-PerCP-Cy5.5, GATA3-PE, Tbet-eFluor® 660, RORγt-PE, CD25-Alexa Fluor® 488, FoxP3-APC, CD11c-PerCP-Cy5.5, MHCII-PE-Cy7, CD11b-PE, and Fixable Viability Dye-eFluor® 780 or matching isotype controls for 30 min at 4 °C. Cells were fixed using fixation buffer (eBioscience) or permeabilized for intracellular staining using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Flow cytometry was performed using FACS Canto II (BD Biosciences), and results were analyzed using FlowLogic Software (Inivai Technologies, Mentone, Australia). To distinguish between negative and positive staining cell populations, fluorescence-minus-one controls were used (after the exclusion of debris, doublets and non-viable cells).

Statistical analysis

Data are presented as mean ± SEM and were analyzed using one- or two-way ANOVA, followed by Bonferroni's multiple comparisons test. As pulmonary IL-5 concentrations did not obtain normality, data were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism software (version 6.07; GraphPad Software, San Diego, CA, USA).

RESULTS

Raw milk prevents HDM-induced AHR and pulmonary inflammation

To assess the effects of raw and heated raw milk on lung function, AHR was measured in response to increasing doses of methacholine. Baseline airway responsiveness tended to be higher in HDM-mice compared to PBS-mice. An increase in AHR was observed in HDM-mice compared to PBS-mice after methacholine exposure. Although intervention with heated raw milk showed no significant effect, increased AHR was prevented by intervention with raw milk (**Figure 2A**). To study the extent of pulmonary inflammation, BALF was examined. The total number of inflammatory cells in the BALF of HDM-mice was significantly higher than in PBS-mice (**Figure 2B**). This was mainly due to an increase in the number of eosinophils (**Figure 2C**), although numbers of lymphocytes, neutrophils, and macrophages were increased as well (**Figures 2D-F**). Raw milk reduced the total number of inflammatory cells in the BALF, which was reflected by lower numbers of eosinophils, lymphocytes, neutrophils, and macrophages (**Figures 2B-F**). Pulmonary inflammation was unaffected by heated raw milk (**Figures 2B-F**).

Raw milk suppresses the frequency of conventional dendritic cells (cDCs) and related CCL17 concentrations in the lung

To determine the effect of raw and heated raw milk on pulmonary cytokines and chemokines, we analyzed supernatants of lung homogenates. IL-33, CCL20, CCL17, and CCL22 concentrations were increased in HDM-mice compared to PBS-mice (**Figures 3A-D**). Neither raw nor heated raw milk were able to affect the concentrations of IL-33, CCL20, and CCL22 (**Figures 3A,B,D**), but both significantly reduced the concentration of CCL17 (**Figure 3C**). Since CCL17 is mainly secreted by $CD11b^+$ cDCs, this DC subtype was analyzed in lung cell suspensions. A higher percentage of $CD11b^+$ cDCs was observed in HDM-mice compared to PBS-mice (**Figure 3E**). While heated raw milk showed no effect on this increase, it was reduced by raw milk (**Figure 3E**).

Percentage of Th2 and Th17 cells in the lung decreases after exposure to raw milk

Lung cell suspensions were analyzed for T cell subsets. The frequency of Th2, Th17, and regulatory T cells (Tregs) was higher in HDM-mice than in PBS-mice (**Figures 4A,B,D**). In line with the reduced concentration of the Th2-attracting chemokine CCL17, the frequency of Th2 cells in the lung was reduced by both raw and heated raw milk (**Figure 4A**). For Th17 cells, a similar effect was observed (**Figure 4B**), while neither milk type affected the percentage of Treg cells (**Figure 4D**). Th1 cells were not increased in HDM-mice compared to PBS-mice and showed no raw milk effect (**Figure 4C**). However, heated raw milk, did reduce the percentage of Th1 cells (**Figure 4C**).

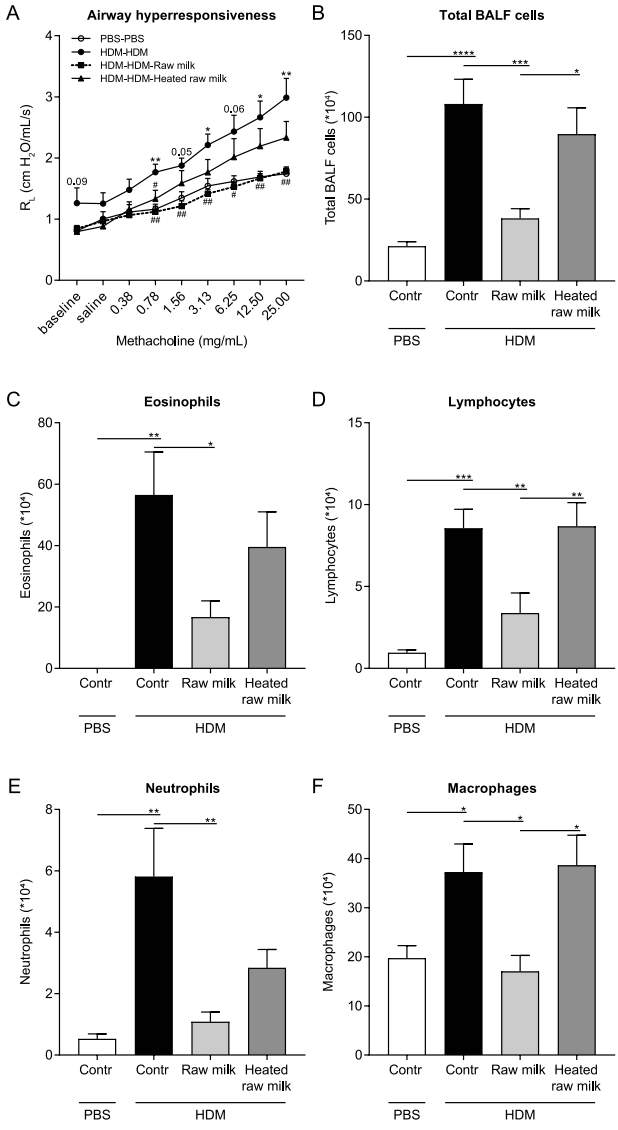


Figure 2. House dust mite (HDM)-induced airway hyperresponsiveness and pulmonary inflammation were prevented by raw milk. (**A**) Lung resistance (R_L) measured after exposure to increasing doses of methacholine. (**B-E**) Differential bronchoalveolar lavage fluid (BALF) cell counts. (**B**) Total BALF cells, (**C**) absolute number of eosinophils, (**D**) lymphocytes, (**E**) neutrophils, and (**F**) macrophages. Data are presented as mean ± SEM. (**A**) *n* = 7-9 mice/group. **P* < 0.05, ***P* < 0.01, compared to PBS-PBS group, $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, compared to HDM-HDM group as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test. (**B-E**) *n* = 8-9 mice/group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test. Contr, mice treated with PBS.

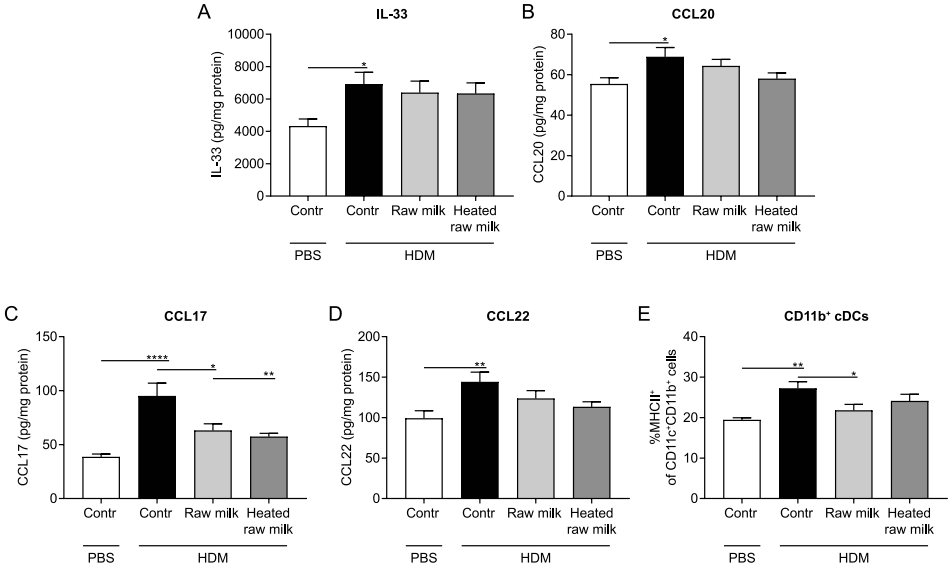


Figure 3. Frequency of CD11b$^+$ conventional dendritic cells (cDCs) and related CCL17 concentrations in the lung were decreased after exposure to raw milk. Levels of epithelial- and DC-derived Th2-polarizing mediators in the lung. (**A**) IL-33, (**B**) CCL20, (**C**) CCL17, and (**D**) CCL22 concentrations were measured in supernatant of lung homogenates (picogram per milligram protein). (**E**) The percentage of CD11b$^+$ cDCs (MHCII$^+$ of CD11c$^+$CD11b$^+$ cells) was analyzed in lung cell suspensions. Data are presented as mean ± SEM, n = 8-9 mice/group. *P < 0.05, **P < 0.01, ****P < 0.0001, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test. Contr, mice treated with PBS.

Raw milk reduces IL-4 and IL-13 production after e*x vivo* restimulation of lung T cells with HDM

To investigate whether the reduction in Th2 and Th17 cell frequency by raw and heated raw milk also affected the production of Th2 and Th17 related cytokines, we measured allergen-specific IL-4, IL-13, and IL-17A secretion in supernatants of lung cell suspensions after *ex vivo* restimulation with HDM. No cytokine production was observed in PBS-mice, whereas HDM-mice showed a significant, HDM-specific, increase in IL-4 and IL-13 (**Figures 5A,B**). Raw milk and heated raw milk reduced the IL-4 and IL-13 concentrations (**Figures 5A,B**). Although IL-17A concentrations were increased in HDM-mice compared to PBS-mice, neither raw nor heated raw milk showed any effect (data not shown).

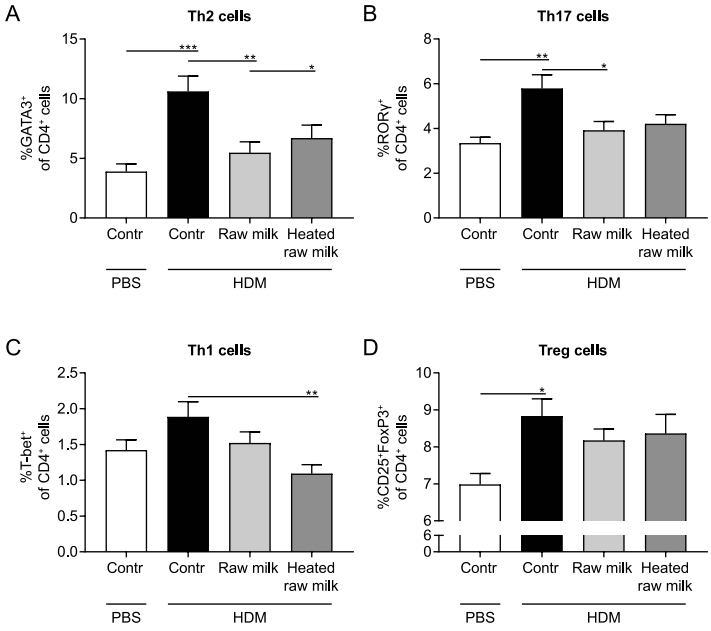


Figure 4. Raw milk suppressed the frequency of Th2 and Th17 cells in the lung. Lung T cell subsets. (**A**) The percentage of Th2 cells (GATA3$^+$ of CD4$^+$ cells), (**B**) Th17 cells (RORγ$^+$ of CD4$^+$ cells), (**C**) Th1 cells (Tbet$^+$ of CD4$^+$ cells), and (**D**) regulatory T cells (Tregs; CD25$^+$FoxP3$^+$ of CD4$^+$ cells) was analyzed in lung cell suspensions. Data are presented as mean ± SEM, n = 8-9 mice/group. *P < 0.05, **P < 0.01, ***P < 0.001, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test. Contr, mice treated with PBS.

Inhibition of pulmonary IL-5 and IL-13 concentrations by raw milk

Supernatants of lung homogenates were furthermore analyzed to investigate whether raw or heated raw milk also reduced local concentrations of type 2 cytokines. IL-5 concentrations did not differ between HDM-mice and PBS-mice, but IL-13 concentrations were increased (**Figures 6A,B**). This increase in IL-13 was reduced by raw milk (**Figure 6B**), which also seemed to suppress IL-5 concentrations (**Figure 6A**). No reduction in local IL-5 or IL-13 concentrations was observed when mice received heated raw milk (**Figures 6A,B**).

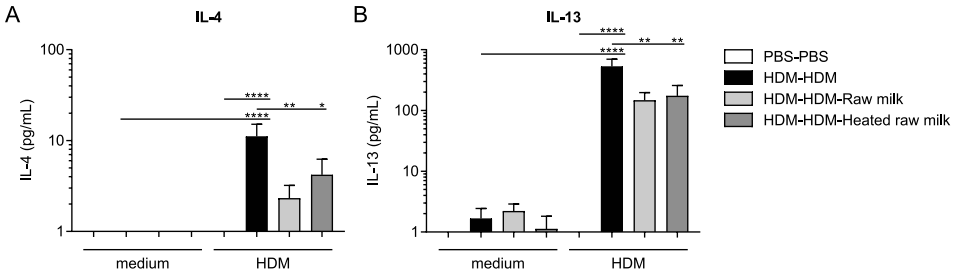


Figure 5. IL-4 and IL-13 production after *ex vivo* restimulation with house dust mite (HDM) was inhibited by raw milk. Lung cell suspensions were *ex vivo* restimulated with medium or HDM for four days (37 °C, 5% CO_2). (**A**) IL-4 (treatment: $P < 0.01$, restimulation: $P < 0.001$, interaction: $P < 0.01$) and (**B**) IL-13 (treatment: $P < 0.01$, restimulation: $P < 0.001$, interaction: $P < 0.01$) concentrations were measured in the supernatants (picogram per milliliter). Data are presented as mean ± SEM, n = 7-9 mice/group. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, as analyzed with two-way ANOVA followed by Bonferroni's multiple comparisons test.

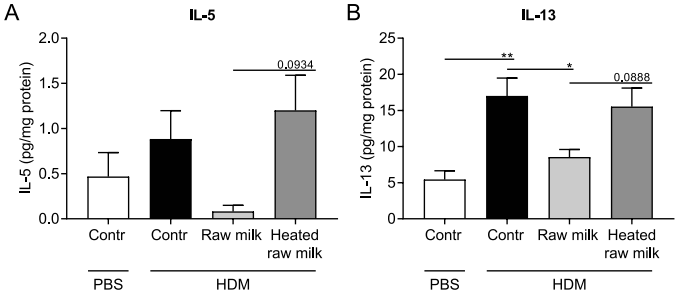


Figure 6. Raw milk reduced pulmonary IL-5 and IL-13 concentrations. Type 2 cytokine concentrations in the lung. (**A**) IL-5 and (**B**) IL-13 concentrations were measured in the supernatant of lung homogenates (picogram per milligram protein). Data are presented as mean ± SEM, n = 7-9 mice/group. *$P < 0.05$, **$P < 0.01$, as analyzed with Kruskal-Wallis followed by Dunn's multiple comparisons test (IL-5) or one-way ANOVA followed by Bonferroni's multiple comparisons test (IL-13). Contr, mice treated with PBS.

DISCUSSION

In the present study, we demonstrated that raw cow's milk consumption prevented the development of asthma in a murine HDM-induced allergic asthma model. Strong protective effects on AHR and airway inflammation were observed which coincided with a reduced type 2 immune response. Heat-treated raw milk showed no asthma-protective effect.

Large-scale epidemiological studies in European populations have consistently shown that growing up on a traditional farm protects against the development of asthma (5-8). These studies invested a lot of effort to determine farm-related exposures contributing to this protective effect. Raw cow's milk consumption is one of the factors repeatedly shown to be protective (9-13). However, all current evidence showing a relationship between raw cow's milk consumption and the prevention of asthma is based on observed associations that do not confirm a causal relationship. To investigate causality, we used a murine HDM-induced allergic asthma model. This model mimics hallmark features of allergic asthma in humans and has clinical relevance due to the use of HDM as aeroallergen (20, 21).

As our data show, and in accordance with existing epidemiological studies, raw cow's milk prevents the development of allergic asthma. Raw milk prevented HDM-induced AHR and abrogated pulmonary inflammation. This protective effect was abolished after heating the milk, which suggests that heat-sensitive milk components play a role in the asthma-protective effect of raw milk. The observed contribution of heat-sensitive components is in line with observations from the GABRIELA study, a large epidemiological study in which an inverse association between the whey fraction of the milk and asthma was found (9). Whey proteins such as α-lactalbumin, β-lactoglobulin, lactoferrin, immunoglobulins, and TGF-β are heat-sensitive and are known to have immunomodulatory effects (22, 23). Follow-up studies with these milk components need to demonstrate whether they also play a role in our model. Another constituent, previously found to be associated with a reduced asthma risk, is the fat content of raw milk (24). Since this fat content is unaffected by heat, it could be responsible for the slight reduction we observed in AHR by mice consuming heated raw milk (0.78 mg/mL of methacholine). However, for the moment, we can only speculate that the fat content in combination with heat-sensitive components is responsible for the asthma-protective effect of raw milk in our study.

To determine how raw milk exerts its effect, we looked at several inflammatory mediators involved in the pathogenesis of allergic asthma. Allergic asthma is generally induced by HDM allergens, and a key trigger in the recognition of HDM is the activation of epithelial cells (25). Epithelial cells are activated when HDM allergens bind to pattern recognition receptors (PRRs), such as TLR4, on their cell membrane. On activation, they produce chemokines and cytokines that attract DCs to the lung and instruct them to induce Th2 immunity (26). Since DCs also express PRRs themselves, they can also be directly activated by HDM allergens (1). Epithelial- (IL-33 and CCL20) and DC-derived (CCL17 and CCL22) mediators were indeed increased after HDM exposure in our model, and the frequency of $CD11b^{+}$ cDCs was also elevated. These results are in line with human studies showing that IL-33, CCL20, CCL17, and CCL22 levels, as well as DC numbers, are elevated in the airways of asthmatic patients compared to healthy controls (27-30). Raw milk did not affect the epithelial mediators but

did reduce the percentage of $CD11b^+$ cDCs and the concentration of CCL17. The $CD11b^+$ cDC subpopulation plays an important role in inducing allergic inflammation via the secretion of CCL17 and CCL22 (31). Both of these chemokines are CCR4 ligands and will attract CCR4-expressing Th2 cells to the lungs, which is essential for the development of allergic asthma (32). The importance of CCL17 and CCL22 is furthermore confirmed in murine asthma models where their neutralization with specific antibodies resulted in attenuation of AHR and airway inflammation (33, 34).

The reduction in the $CD11b^+$ cDC-derived CCL17 concentration by raw milk may have resulted in a reduced Th2 cell influx. Indeed, the frequency of $GATA3^+CD4^+$ Th2 cells in the lung was decreased by raw milk. GATA3 expression is known to be markedly increased in T cells derived from the airways of asthmatic patients compared to healthy individuals (35). It is essential not only for the differentiation of naïve T cells into Th2 cells but also for the secretion of Th2-related cytokines (IL-4, IL-5, and IL-13) (36, 37). In addition to reducing Th2 cell frequency, raw milk also reduced the percentage of Th17 cells in the lung, which mainly recruit and activate neutrophils and are known to be increased in asthmatic patients (38). This reduction in Th17 cells indeed coincided with a reduced neutrophil influx to the lung. While the exact contribution of Th17 cells in allergic asthma remains to be elucidated, blocking Th17 immunity as a therapy is gaining interest (39, 40). The reduction of Th2 and Th17 cells by raw milk was not accompanied by a shift towards a more Th1 or Treg immune response.

In accordance with the reduced Th2 cell influx, raw milk also reduced concentrations of Th2-related cytokines. IL-4 and IL-13 production was suppressed after *ex vivo* restimulation of lung T cells with HDM. In addition, local pulmonary IL-13 concentrations were inhibited and a similar pattern was observed for IL-5 (**Figure 7**). Together, these type 2 cytokines are responsible for the salient features of allergic asthma (3, 41).

In contrast to raw milk, heated raw milk was not able to prevent the development of asthma. Even though it reduced pulmonary CCL17 concentrations, the frequency of Th2 and Th17 cells in the lung, and the IL-4 and IL-13 production by lung T cells after *ex vivo* restimulation with HDM, which is potentially beneficial in allergic diseases, this did not result in reduced airway inflammation in our study. Heated raw milk did not affect local pulmonary IL-5 and IL-13 concentrations. This discrepancy in the effects on type 2 cytokines might be explained by the fact that local levels can also be produced by immune cells other than T cells. It is well known that type 2 cytokines are produced not only by Th2 cells but also by type 2 innate lymphoid cells, eosinophils, basophils, and mast cells (42). Since raw milk suppressed local type 2 cytokine levels, this implies that a reduction in locally produced IL-5 and IL-13 is essential to the preventive effects of raw milk. Furthermore, it suggests that only raw milk affects these other cells types, but this needs to be confirmed in future studies (**Figure 7**).

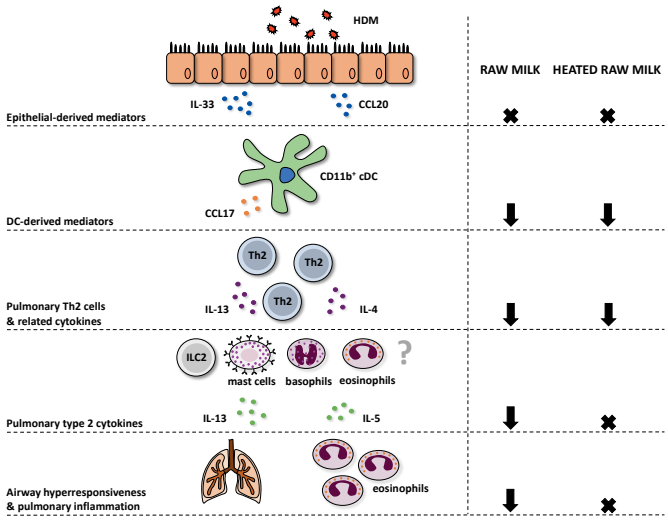


Figure 7. Schematic overview of the effects of raw and heated raw milk on allergic asthma. Following exposure to house dust mite (HDM), lung epithelial cells produce chemokines, like CCL20, that attract immature preconventional dendritic cells (cDCs) to the lung, and cytokines, like IL-33, that activate CD11b⁺ cDCs. Concentrations of these epithelial-derived mediators in the lung were unaffected by raw and heated raw milk. Activated lung CD11b⁺ cDCs subsequently migrate to the mediastinal lymph nodes where they differentiate naïve T cells into Th2 cells. These Th2 cells migrate back to the lung mucosal tissue in response to CCL17 and CCL22 produced by poorly migratory DCs. Lung CCL17 concentration, as well as lung Th2 cell frequency, was reduced by both milk types. On subsequent HDM challenges, Th2 cells will start to produce IL-4, IL-5, and IL-13. The concentration of these Th2-related cytokines was also suppressed by both milk types after *ex vivo* restimulation of lung T cells with HDM. However, pulmonary concentrations of these cytokines, measured in lung homogenates, were only reduced by raw milk. Since not only Th2 cells but also type 2 innate lymphoid cells (ILC2), mast cells, basophils, and eosinophils can produce these type 2 cytokines, it suggests that only raw milk affects these other cell types. Eventually, only raw milk prevented airway hyperresponsiveness and pulmonary inflammation indicating the importance of reduced local type 2 cytokine levels.

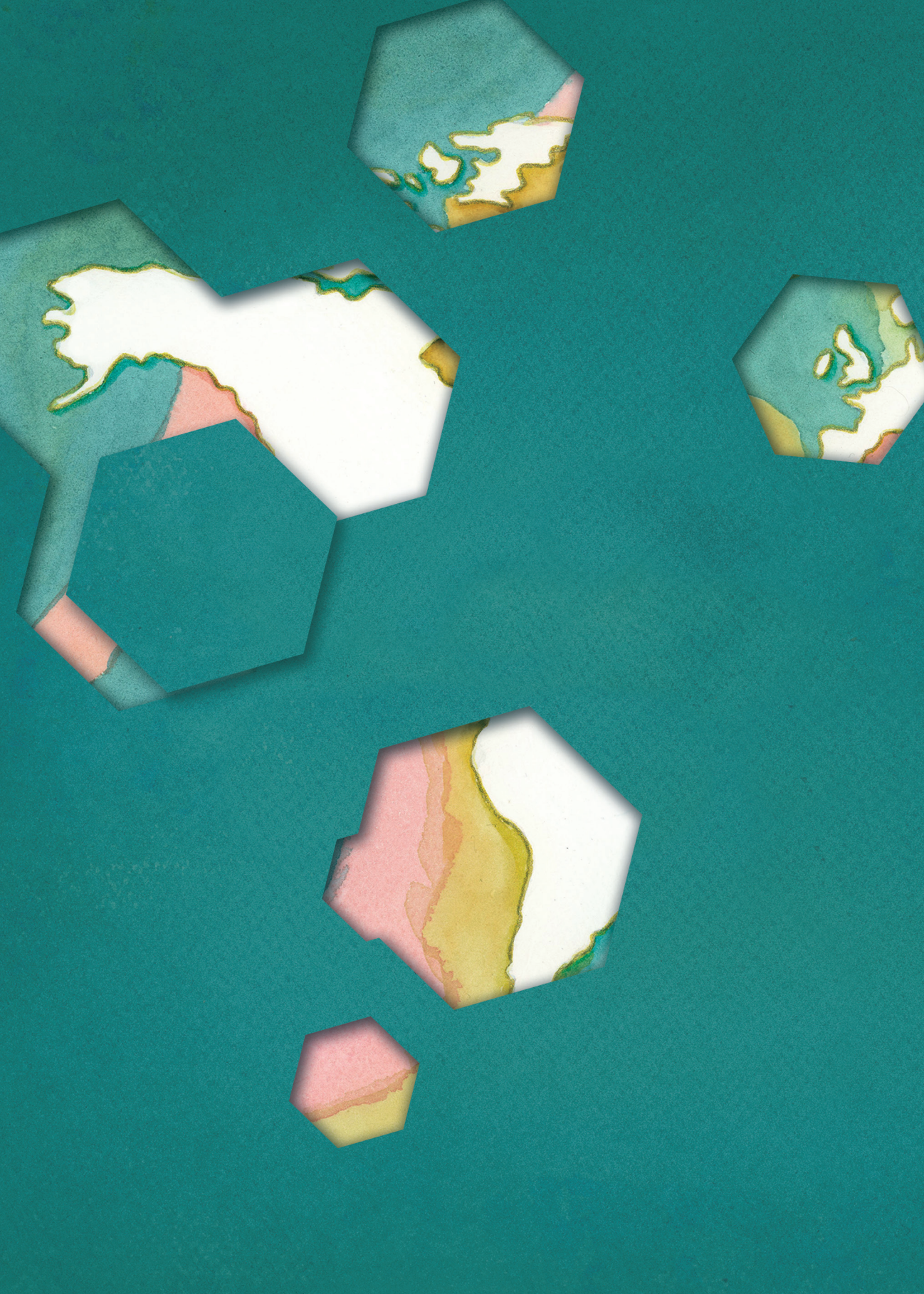
The promising results of this study suggest that there is a causal relationship between raw cow's milk consumption and the prevention of allergic asthma. The protective effect seems to start as early as the DC level, where mediators priming Th2 immunity were suppressed. However, suppression of local type 2 cytokine levels seems to be crucial. This study supports current epidemiological findings and emphasizes the need for minimally processed, safe milk. By comparing raw and heated raw milk, a first attempt was made to pinpoint the milk components underlying the asthma-protective effect of raw milk. Heat-sensitive milk components were found to be responsible. However, future research should aim at elucidating the specific heat-sensitive milk components involved and their underlying mechanism.

REFERENCES

1. Lambrecht BN and Hammad H. The immunology of asthma. *Nat Immunol* (2015) 16(1): 45-56.
2. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* (2012) 18(5): 673-83.
3. Hammad H and Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* (2008) 8(3): 193-204.
4. Alfvén T, Braun-Fahrländer C, Brunekreef B, von Mutius E, Riedler J, Scheynius A, *et al*. Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle - The PARSIFAL study. *Allergy* (2006) 61(4): 414-21.
5. Kilpeläinen M, Terho EO, Helenius H, and Koskenvuo M. Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* (2000) 30(2): 201-8.
6. Riedler J, Eder W, Oberfeld G, and Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin Exp Allergy* (2000) 30(2): 194-200.
7. von Ehrenstein OS, von Mutius E, Illi S, Baumann L, Böhm O, and von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* (2000) 30(2): 187-93.
8. Ernst P and Cormier Y. Relative scarcity of asthma and atopy among rural adolescents raised on a farm. *Am J Resp Crit Care* (2000) 161(5): 1563-6.
9. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
10. Waser M, Michels KB, Bieli C, Flöistrup H, Pershagen G, von Mutius E, *et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
11. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
12. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
13. Riedler J, Braun-Fahrländer C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
14. von Mutius E and Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* (2010) 10(12): 861-8.
15. Michalski MC. On the supposed influence of milk homogenization on the risk of CVD, diabetes and allergy. *Br J Nutr* (2007) 97(4): 598-610.
16. Braun-Fahrländer C and von Mutius E. Can farm milk consumption prevent allergic diseases? *Clin Exp Allergy* (2011) 41(1): 29-35.
17. Kool M, Willart MA, van Nimwegen M, Bergen I, Pouliot P, Virchow JC, *et al*. An unexpected role for uric acid as an inducer of T helper 2 cell immunity to inhaled antigens and inflammatory mediator of allergic asthma. *Immunity* (2011) 34(4): 527-40.
18. Verheijden KA, Henricks PA, Redegeld FA, Garssen J, and Folkerts G. Measurement of airway function using invasive and non-invasive methods in mild and severe models for allergic airway inflammation in mice. *Front Pharmacol* (2014) 5: 190.
19. Verheijden KA, Willemsen LE, Braber S, Leusink-Muis T, Delsing DJ, Garssen J, *et al*. Dietary galacto-oligosaccharides prevent airway eosinophilia and hyperresponsiveness

in a murine house dust mite-induced asthma model. *Respir Res* (2015) 16: 17.
20. Nials AT and Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech* (2008) 1(4-5): 213-20.
21. Gregory LG and Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol* (2011) 32(9): 402-11.
22. Chatterton DE, Nguyen DN, Bering SB, and Sangild PT. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int J Biochem Cell Biol* (2013) 45(8): 1730-47.
23. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
24. Brick T, Schober Y, Böcking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-706 e13.
25. Lambrecht BN and Hammad H. The airway epithelium in asthma. *Nat Med* (2012) 18(5): 684-92.
26. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, and Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* (2009) 15(4): 410-6.
27. Saglani S, Lui S, Ullmann N, Campbell GA, Sherburn RT, Mathie SA, *et al*. IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma. *J Allergy Clin Immunol* (2013) 132(3): 676-85.
28. Pichavant M, Charbonnier AS, Taront S, Brichet A, Wallaert B, Pestel J, *et al*. Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. *J Allergy Clin Immunol* (2005) 115(4): 771-8.
29. Pilette C, Francis JN, Till SJ, and Durham SR. CCR4 ligands are up-regulated in the airways of atopic asthmatics after segmental allergen challenge. *Eur Respir J* (2004) 23(6): 876-84.
30. Möller GM, Overbeek SE, Van Helden-Meeuwsen CG, Van Haarst JMW, Prens EP, Mulder PG, *et al*. Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled corticosteroids. *Clin Exp Allergy* (1996) 26(5): 517-24.
31. Gill MA. The role of dendritic cells in asthma. *J Allergy Clin Immunol* (2012) 129(4): 889-901.
32. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* (2008) 118(11): 3546-56.
33. Kawasaki S, Takizawa H, Yoneyama H, Nakayama T, Fujisawa R, Izumizaki M, *et al*. Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J Immunol* (2001) 166(3): 2055-62.
34. Gonzalo JA, Pan Y, Lloyd CM, Jia GQ, Yu G, Dussault B, *et al*. Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J Immunol* (1999) 163(1): 403-11.
35. Caramori G, Lim S, Ito K, Tomita K, Oates T, Jazrawi E, *et al*. Expression of GATA family of transcription factors in T-cells, monocytes and bronchial biopsies. *Eur Respir J* (2001) 18(3): 466-73.
36. Rayees S, Malik F, Bukhari SI, and Singh G. Linking GATA-3 and interleukin-13: implications in asthma. *Inflamm Res* (2014) 63(4): 255-65.
37. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* (2008) 8(3): 183-92.
38. Zhao Y, Yang J, Gao YD, and Guo W. Th17 immunity in patients with allergic asthma. *Int Arch Allergy Immunol* (2010) 151(4): 297-307.
39. Cosmi L, Liotta F, Maggi E, Romagnani S, and Annunziato F. Th17 cells: new players in asthma pathogenesis. *Allergy* (2011) 66(8): 989-98.
40. Ivanov S and Linden A. Interleukin-17 as a drug target in human disease. *Trends Pharmacol Sci* (2009) 30(2): 95-103.

41. Kudo M, Ishigatsubo Y, and Aoki I. Pathology of asthma. *Front Microbiol* (2013) 4: 263.
42. Schuijs MJ, Willart MA, Hammad H, and Lambrecht BN. Cytokine targets in airway inflammation. *Curr Opin Pharmacol* (2013) 13(3): 351-61.



CHAPTER 4

Raw cow's milk reduces allergic symptoms in a murine model for food allergy – A potential role for epigenetic modifications

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ABSTRACT

Epidemiological studies identified raw cow's milk consumption as an important environmental exposure that prevents allergic diseases. In the present study, we investigated whether raw cow's milk has the capacity to induce tolerance to an unrelated, non-milk, food allergen. Histone acetylation of T cell genes was investigated to assess potential epigenetic regulation. Female C3H/HeOuJ mice were sensitized and challenged with ovalbumin. Prior to sensitization, mice were treated with raw milk, processed milk, or PBS for eight days. Allergic symptoms were assessed after challenge and histone modifications in T cell-related genes of splenocyte-derived $CD4^+$ T cells and the mesenteric lymph nodes were analyzed after milk exposure and after challenge. Unlike processed milk, raw milk decreased allergic symptoms. After raw milk exposure, histone acetylation of Th1-, Th2- and regulatory T cell-related genes of splenocyte-derived $CD4^+$ T cells was higher than after processed milk exposure. After allergy induction, this general immune stimulation was resolved and histone acetylation of Th2 genes was lower when compared to processed milk. Raw milk reduces allergic symptoms to an unrelated, non-milk, food allergen in a murine model for food allergy. The activation of T cell-related genes could be responsible for the observed tolerance induction, suggesting that epigenetic modifications contribute to the allergy-protective effect of raw milk.

INTRODUCTION

Allergic diseases are a growing public health concern. In the previous decades, their prevalence has increased to such an extent that, nowadays, 20 to 30% of the world's population is suffering from some form of allergic disease (1). With a severe impact on quality of life and extensive health care costs, the vast prevalence of allergic diseases has major socio-economic consequences (2). Unfortunately, to date, there is neither a cure nor an effective and safe treatment. Allergy management focuses on allergen avoidance and symptomatic treatment with the self-administration of epinephrine in the case of systemic anaphylaxis upon accidental exposure.

Even though there are no effective preventive approaches for allergic diseases, there seems to be a natural solution. Several epidemiological studies have shown that children growing up on a farm have a reduced risk of developing asthma and allergies compared to children living in the same rural area but not growing up on a farm (3-7). This protective 'farm effect' was demonstrated in many populations and persisted into adult life (8). Farm exposures that were associated with this allergy-protective effect appeared to be contact with livestock and animal feed, exposure to stables and barns, and consumption of raw, unprocessed, cow's milk (9-11). Especially, the consumption of raw cow's milk is of importance, since its protective effect was found to be independent of farm status, giving it the potential to confer protection for a general, non-farming, population (9, 10, 12, 13). Recently, these epidemiological findings were confirmed by showing a causal relationship between raw cow's milk consumption and the prevention of allergic asthma in a murine model (14).

How raw cow's milk can be allergy protective is currently still unclear. Neither the protective raw milk constituents nor the underlying mechanisms are known. Heat-sensitive milk components, like immunoglobulins, lactoferrin, alkaline phosphatase, TGF-β, microRNAs, etc., are likely candidates, since epidemiological as well as preclinical studies have shown that milk processing, and particularly heating, abolishes the allergy-protective effect of raw cow's milk consumption (13-16). However, the actual bioactive component(s) involved remain to be elucidated. Regarding the underlying mechanisms, several of the bioactive components that are present in raw milk are theoretically able to create a tolerogenic environment by, for example, promoting regulatory T cell development, enhancing epithelial barrier function, and modulating the gut microbiome, however, none of these effects were actually investigated after drinking raw milk (17, 18).

An emerging field is the contribution of epigenetic modifications in regulating the development of allergic diseases. Allergic diseases are the result of a complex interplay between genes and environmental factors. These environmental factors can influence gene expression via epigenetic mechanisms, such as DNA methylation and histone modifications (19, 20). Epigenetic

modifications are reversible, and they affect the accessibility of the DNA to transcription enzymes, thereby regulating gene expression (19). Environmental factors and components recently gaining interest in this regard are microbes, obesity, stress, and tobacco smoke, but it has also been suggested that nutrients might exert their effects through epigenetic mechanisms (19, 21). This indicates that epigenetic regulation might also be involved in the allergy-protective effect of raw cow's milk consumption.

Before certified raw cow's milk (raw cow's milk obtained from a farm that is legally allowed to sell raw milk (22)) can become part of a preventive approach for allergic diseases, compelling evidence that thoroughly investigates components and mechanisms that are involved is needed. As a first step, the many epidemiological studies showing an allergy-protective effect of raw cow's milk consumption need to be strengthened by causal evidence. In a previous study, we were able to show causality in a murine house dust mite-induced asthma model (14). With the current research, we aimed to assess whether raw cow's milk has the capacity to induce tolerance to an unrelated, non-milk, food allergen. Besides, we studied the contribution of epigenetic regulation by assessing histone acetylation of T cell-related genes, as a potential mechanism underlying the protective effects.

MATERIALS & METHODS

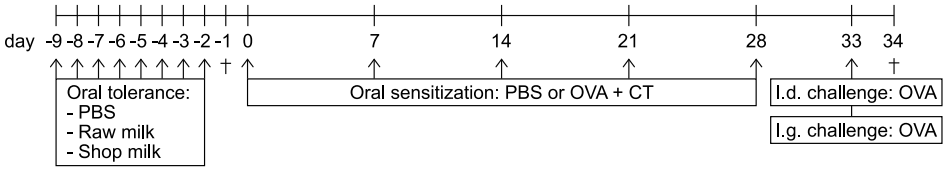
Animals

Specific pathogen-free, three- to five-week-old, female C3H/HeOuJ mice were purchased (Charles River Laboratories, Sulzfeld, Germany) and were randomly allocated to the control or experimental groups. The mice were housed in filter-topped makrolon cages (one cage/group, *n* = 6-8/cage) with standard chip bedding, Kleenex tissues, and a plastic shelter on a 12 h light/dark cycle with unlimited access to food ('Rat and Mouse Breeder and Grower Expanded'; Special Diet Services, Witham, UK) and water at the animal facility of Utrecht University (Utrecht, The Netherlands). All animal procedures were approved by the Ethical Committee for Animal Research of the Utrecht University and were complied with the European Directive on the protection of animals used for scientific purposes (DEC 2014.II.12.107 & AVD108002015346).

Experimental design – Tolerance induction, sensitization and challenges

After an acclimatization period of one week, mice were orally treated (i.e., intragastrically [i.g.] by using a blunt needle) with 0.5 mL certified raw, unprocessed, cow's milk (Hof Dannwisch, Horst, Germany), processed shop milk (full fat milk, 3.5%; EDEKA, Germany), or PBS (as a control) for eight consecutive days (days -9 to -2). Following this oral tolerance induction period, mice were sensitized i.g. once a week for five weeks to the hen's egg protein ovalbumin (OVA; 20 mg/0.5 mL PBS; grade V; Sigma-Aldrich, Zwijndrecht, The Netherlands) using 10 µg cholera

toxin (CT; List Biological Laboratories, Campbell, CA, USA) as an adjuvant (days 0, 7, 14, 21, 28; *n* = 8/group). Sham-sensitized control mice (*n* = 6) received CT alone (10 µg/0.5 mL PBS). Five days after the last sensitization (day 33), all mice were intradermally (i.d.) challenged in both ear pinnae with 10 µg OVA in 20 µL PBS to determine acute allergic symptoms. Mice were subsequently i.g. challenged (7 h after the i.d. challenge) with 50 mg OVA in 0.5 mL PBS. Sixteen hours later (day 34), a blood sample was taken via cheek puncture and mice were killed by cervical dislocation. The spleens were then collected for *ex vivo* analysis. Additional groups of mice (*n* = 6/group) were used in a follow-up experiment to assess the involvement of epigenetic regulation. These mice were killed by cervical dislocation either one day after the oral tolerance induction period (day -1) or one day after both challenges (day 34). **Figure 1** shows a schematic representation of the experimental timeline.



Group	Tolerance	Sensitization	Challenge
PBS	PBS	PBS + CT	OVA
OVA	PBS	OVA + CT	OVA
Raw milk	Raw milk	OVA + CT	OVA
Shop milk	Shop milk	OVA + CT	OVA

Figure 1. Schematic representation of the experimental timeline. For epigenetic measurements, additional groups of mice were killed after the tolerance induction period (day -1) and after both challenges (day 34; as indicated by †). OVA, ovalbumin; CT, cholera toxin; i.d., intradermal; i.g., intragastric.

Assessment of the acute allergic response

To determine the severity of the acute allergic symptoms, the acute allergic skin response, anaphylactic shock symptoms, and body temperature were evaluated by a researcher blinded to treatment upon i.d. challenge with OVA (10 µg OVA/20 µL PBS) in the ear pinnae of both ears. The acute allergic skin response was measured as Δ ear swelling (µm) by subtracting the mean ear thickness before i.d. challenge from the mean ear thickness 1 h after i.d. challenge. Ear thickness at both timepoints was measured in duplicate for each ear using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). To perform the i.d. challenge as well as the ear measurements, mice were anesthetized using inhalation of isoflurane (Abbott, Breda, The Netherlands). The severity of anaphylactic shock symptoms was determined 30 min after i.d. challenge by using a previously described, validated, scoring table (23). Body temperature was also measured 30 min after i.d. challenge (using a rectal thermometer) to monitor the anaphylactic shock-induced drop in body temperature.

Detection of OVA-specific IgE and mMCP-1 in serum

Blood was collected via cheek puncture 16 h after i.g. challenge, centrifuged at 10,000 rpm for 10 min, and serum was stored at -20 °C until the analysis of OVA-specific IgE and mucosal mast cell protease-1 (mMCP-1) levels by means of ELISA. OVA-specific IgE titers were detected, as described previously (24). Levels are expressed in arbitrary units (AU), which were calculated based on a titration curve of pooled sera serving as an internal standard. The concentrations of mMCP-1 were determined using a mMCP-1 Ready-SET-Go!® ELISA (eBioscience, Breda, The Netherlands), according to the manufacturer's protocol.

***Ex vivo* OVA-specific stimulation of splenocytes for cytokine measurements**

Spleens were collected and homogenized using a syringe and a 70 µm nylon cell strainer. The obtained single cell splenocyte suspensions were incubated with lysis buffer (8.3 g NH_4Cl, 1 g KHC_3O, and 37.2 mg EDTA dissolved in 1 L demi water, filter sterilized) to remove red blood cells and then resuspended in RPMI 1640 medium (Lonza, Verviers, Belgium), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands), penicillin (100 U/mL)/streptomycin (100 µg/mL; Sigma-Aldrich), and β-mercaptoethanol (20 µM; Thermo Fisher Scientific, Paisley, Scotland). The splenocytes (8×10^5 cells/well) were cultured in U-bottom culture plates (Greiner, Frickenhausen, Germany), either with medium or with 50 µg/mL OVA for four days at 37 °C, 5% CO_2. The supernatants were collected and stored at -20 °C until cytokine analysis. The concentrations of IL-5 and IL-13 were measured by means of ELISA, as described elsewhere (25). The concentrations of IFNγ, IL-10, and IL-17 were measured using a Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, Alphen aan de Rijn, The Netherlands), according to the manufacturer's instructions. The results were obtained using FACS Canto II and analyzed with FCAP Array Software, version 3.0 (BD Biosciences). Cytokine concentrations measured after medium stimulation were subtracted from cytokine concentrations measured after OVA stimulation to determine the OVA-specific cytokine response. A zero was entered when this resulted in a negative value.

Chromatin immunoprecipitation to determine histone acetylation status in splenocyte-derived $CD4^+$ T cells and mesenteric lymph nodes (MLN)

At day -1 (after the tolerance induction period) and at day 34 (after both challenges), $CD4^+$ T cells were isolated from splenocytes of raw milk- and shop milk-treated mice using MACS, according to the manufacturer's instructions (Miltenyi Biotec, Leiden, The Netherlands). Isolated $CD4^+$ T cells were frozen with 15% dimethyl sulphoxide (DMSO; Sigma-Aldrich) in heat-inactivated FBS (Bodinco) and then stored in liquid nitrogen until further analysis. For the MLN, the entire tissue, containing a full population of the MLN cells, was frozen in 15% DMSO-FBS and stored in liquid nitrogen until further analysis. Detailed methodology of chromatin immunoprecipitation, followed by real-time polymerase chain reaction (ChIP-qPCR), along with

its thoughtful validations, were previously described in detail (26). In brief, the MLN tissues were first smashed through a mesh, washed with 1 mL of PBS (Sigma-Aldrich), and centrifuged at 8000 rpm for 5 min at 4 °C. The pellet was then resuspended in 1 mL of warm PBS. The cross-linking of the cells was performed by incubating the cells with paraformaldehyde (PFA; Carl Roth GmbH, Karlsruhe, Germany) to a final concentration of 1% for 8 min at room temperature. The reaction was quenched by adding glycine to a final concentration of 125 mM (Carl Roth GmbH). After centrifugation at 8000 rpm for 5 min at 4 °C and washing with cold PBS, the samples were subjected to 20 min of incubation with lysis buffer I (**Table S1**) at 4 °C. Lysis buffer II (**Table S1**) was added with 1% sodium dodecyl sulfate (SDS; Carl Roth GmbH) for 5 min at 4 °C. Shearing of the DNA-protein complexes with the Bioruptor (Diagenode, Liège, Belgium) was conducted afterwards using 30 cycles (30 s on, 30 s off) for $CD4^+$ T cells and 40 cycles (40 s on, 40 s off) for MLN cells. Finally, the interfering debris was removed by centrifugation at 15,000 rpm for 15 min at 4 °C. Sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) were first washed with lysis buffer II with 0.1% SDS. Following centrifugation at 3000 rpm for 2 min at room temperature, the beads were blocked with 1 mg/mL bovine serum albumin (BSA; Sigma-Aldrich) and 40 µg/mL salmon sperm DNA (Sigma-Aldrich) overnight at 4 °C. After washing the prepared beads with lysis buffer II with 0.1% SDS and centrifugation at 3000 rpm for 5 min at 4 °C, 30 µL of beads slurry per immunoprecipitation (IP) per number of samples were stored at 4 °C for the next day. To perform chromatin preclearing, 20 µL of beads slurry per antibody were added to the previously cross-linked chromatin samples, incubated with rotation for 2 h at 4 °C, and then centrifuged at 8000 rpm for 5 min at 4 °C. To the rest of the beads, 500 µL of lysis buffer II with 0.1% SDS and 1 µg of unspecific IgG (Abcam, Cambridge, UK) per sample were added and then incubated with rotation for 1 h at 4 °C. After washing three times with lysis buffer II with 0.1% SDS, 20 µL of the IgG-coupled beads were added to the precleared chromatin, incubated with rotation for 2 h at 4 °C, and then centrifuged at 8000 rpm for 5 min at 4 °C. Ten percent of the resulting supernatant containing chromatin was stored as the input control. The rest was divided into equal parts, to which 4 µg of either H3 or H4 (Millipore, Darmstadt, Germany) or 0.5 µg of IgG (Abcam) was added. The samples were then incubated at 4 °C overnight. Thirty microliter of the blocked beads slurry kept aside before, was added to each IP, and incubated for 2 h at 4 °C. After centrifugation at 8000 rpm for 5 min at 4 °C, the beads were washed twice with wash buffer I, twice with lysis buffer II, three times with wash buffer III (**Table S1**), and then twice with TE buffer with pH 8.0 (**Table S1**). The elution of the chromatin was performed by adding 500 µL of elution buffer (**Table S1**) to the sepharose beads, vortexing and incubating with rotation for 30 min. After centrifugation at 8000 rpm for 2 min at 4 °C, the supernatants containing each IP, as well as the input controls, were mixed with 20 µL of 5 M NaCl, 10 µL of 0.5 M EDTA (Sigma-Aldrich), 20 µL of 1 M Tris-HCl (pH 7.2), 1 µL of Protease K (20 mg/mL; Sigma-Aldrich), and 1 µL of RNAse A (10 mg/mL; Sigma-Aldrich) per sample. All of the samples were incubated at 55 °C for 3 h and then at 65 °C overnight. Afterwards, DNA was purified using the QIAquick

PCR purification kit (Qiagen, Hilden, Germany). The purified DNA was subjected to qPCR that was performed with specific mouse gene promoter primers (**Table S2**) and Rotor-Gene SYBR Green PCR Kit (Qiagen), performed on Rotor-Gene Q (Qiagen). We were unfortunately unable to successfully amplify RORγ from H4-immunoprecipitated MLN DNA despite of two rounds of repetition, most probably due to the presence of a specific inhibition of this PCR in this batch of the samples. Percent enrichment to the input was calculated using the following formula: $\%\ \text{enrichment} = 100 \times 2^{[(\text{CT input}-3.3)-\text{CT sample}]}$. Subsequently, the % enrichment of the isotype (IgG) control was subtracted from % enrichments that were obtained for specific antibodies. For final normalization, to further eliminate the variation caused by sample handling, such value obtained for each specific gene was divided by that of the positive control gene ribosomal protein L32 (RPL32) (26, 27).

Statistical analysis

Experimental results are expressed as mean ± SEM or as individual data points or box-and-whisker Tukey plots when data were not normally distributed and analyzed using GraphPad Prism software (version 7.03; GraphPad Software, San Diego, CA, USA). Differences between pre-selected groups were statistically determined using one-way ANOVA, followed by Bonferroni's multiple comparisons test. Square root transformation was applied to mMCP-1 concentrations prior to ANOVA analysis. Anaphylactic shock scores and OVA-specific IgE levels were analyzed using the Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups. For histone acetylation and cytokine concentrations, differences between groups were statistically determined with an unpaired two-tailed Student's *t*-test. Welch's correction was used when the group variances were not equal. When data did not obtain normality, a Mann-Whitney test was performed. The results were considered to be statistically significant when $P < 0.05$.

RESULTS

Raw milk reduces OVA-induced allergic symptoms

To assess whether raw, unprocessed, cow's milk has the capacity to induce tolerance to an unrelated, non-milk, food allergen, mice were orally treated for eight consecutive days with raw cow's milk before being sensitized and challenged with OVA. Upon i.d. challenge with OVA, acute allergic symptoms were, as expected, increased in OVA-sensitized allergic mice when compared to PBS-sensitized control mice. This was illustrated by an increased acute allergic skin response, increased anaphylactic shock symptoms, and an anaphylactic shock-induced drop in body temperature (**Figures 2A-C**). Treating mice with raw milk prior to OVA-sensitization reduced acute allergic symptoms when compared to PBS-treated allergic mice. The allergic skin response and anaphylactic shock symptoms were decreased and the

body temperature of these mice remained high (**Figures 2A-C**). To determine whether this allergy-suppressive effect is abolished upon milk processing, mice were also treated with a processed, shop, milk. Treatment with this shop milk did not confer protection against allergic symptoms (**Figures 2A-C**).

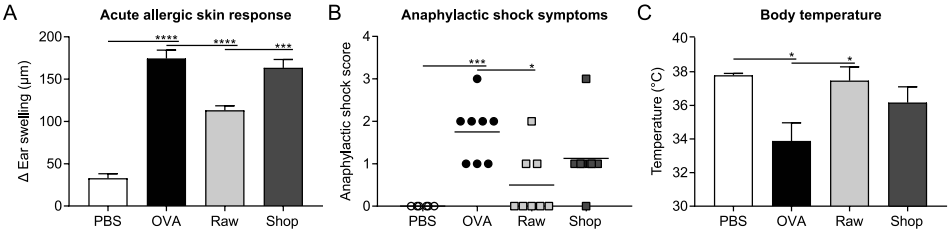


Figure 2. Reduced acute allergic symptoms upon ovalbumin (OVA) challenge in mice treated with raw milk. (**A**) The acute allergic skin response measured as Δ ear swelling 1 h after intradermal (i.d.) challenge. (**B**) Anaphylactic shock scores and (**C**) body temperature determined 30 min after i.d. challenge. Data are presented as mean ± SEM for the acute allergic skin response and body temperature and as individual data points for anaphylactic shock scores, n = 6 in PBS group and n = 8 in all other groups. *P < 0.05, ***P < 0.001, ****P < 0.0001, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups (**A, C**) or Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups (**B**). OVA, ovalbumin; raw, raw cow's milk; shop, shop milk; i.d., intradermal.

OVA-specific IgE levels and mucosal mast cell degranulation are not affected by raw milk exposure

Since food allergens mainly induce type I hypersensitivity reactions, which are characterized by the production of allergen-specific IgE antibodies, the effect of raw and shop milk on serum OVA-IgE levels was investigated. Serum OVA-IgE levels were elevated in OVA-sensitized mice when compared to PBS-sensitized mice (**Figure 3A**). Even though OVA-IgE levels were not significantly affected by exposure to both milk types, they did follow a similar pattern as the acute allergic symptoms, with low OVA-IgE levels in the raw milk group and higher levels in the shop milk group (**Figure 3A**). In addition, serum mMCP-1 concentration, as a marker for mucosal mast cell degranulation, was measured. mMCP-1 concentrations were increased in OVA-sensitized mice when compared to PBS-sensitized mice but were unaffected by treatment with raw or shop milk (**Figure 3B**).

Raw milk treatment initially increases histone acetylation of several T cell subset genes, while after both challenges it specifically reduces Th2-related gene acetylation

Environmental factors might interact with genes that are involved in allergy development via epigenetic regulation. To determine whether epigenetic modifications contribute to the allergy-protective effect of raw cow's milk consumption, histone acetylation (associated with higher

gene expression) at selected Th1-, Th2-, Th17-, and regulatory T cell (Treg)-specific genes of splenocyte-derived $CD4^+$ T cells was assessed. Surprisingly, histone H4 acetylation of Th2-related genes (GATA3, IL-4, IL-5, and IL-13) was higher after eight days of raw milk exposure when compared to shop milk exposure (day -1; **Figure 4A**). Raw milk exposure also increased the histone acetylation of T-bet and tended to increase the histone acetylation of FoxP3 (day -1), indicating a type of general immune stimulation (**Figure 4D**). After both challenges (day 34), this general immune stimulation that was induced by raw milk was resolved and the histone acetylation of Th2 genes was lower as compared to shop milk (**Figures 4B,E**). Furthermore, the histone acetylation pattern of Th2-related genes is visualized by the raw milk/shop milk ratio, which shifted from in favor of raw milk after tolerance to in favor of shop milk after challenge (**Figure 4C**). A similar pattern was observed for IL-17, whereas the raw milk/shop milk ratio for Th1- and Treg-specific genes remained in favor of raw milk throughout the experiment (**Figure 4F**). For histone H3, the acetylation patterns were comparable (**Figure S1**).

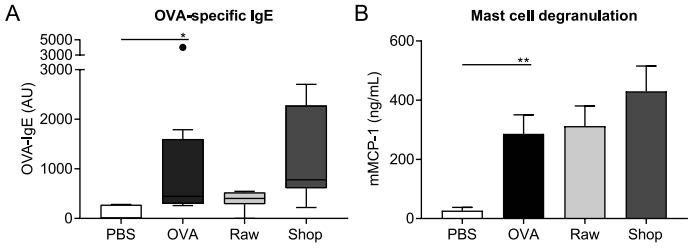


Figure 3. Raw milk treatment did not affect ovalbumin (OVA)-specific IgE levels and mucosal mast cell protease-1 (mMCP-1) concentrations. (**A**) OVA-specific IgE levels and (**B**) mMCP-1 concentrations measured in serum 16 h after intragastric challenge. Data are expressed as box-and-whisker Tukey plot (in which outliers are shown as separately plotted points) for OVA-specific IgE levels and as mean ± SEM for mMCP-1 concentrations, $n = 6$ in PBS group and $n = 8$ in all other groups. $^*P < 0.05$, $^{**}P < 0.01$, as analyzed with Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups (**A**) or one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups (**B**). OVA, ovalbumin; AU, arbitrary units; raw, raw cow's milk; shop, shop milk; mMCP-1; mucosal mast cell protease-1.

Systemically observed acetylation profile of Th2-related genes induced by raw milk also visible locally

To determine whether the systemically observed alterations in histone H4 acetylation of T cell genes induced by raw milk are also visible locally, MLN were analyzed. Despite being less strong, the shift in acetylation of Th2-related genes was also evident in the MLN (**Figures 5A-C**). Raw milk exposure for eight days led to higher acetylation of Th2-related cytokine genes (IL-4, IL-5, and IL-13) when compared to shop milk (day -1), while a lower acetylation of these genes was observed after both challenges (day 34; **Figures 5A,B**). For GATA3, histone acetylation was lower in the raw milk group after tolerance, as well as after the challenges (**Figures 5A,B**).

The general immune stimulation, as observed after tolerance in CD4⁺ T cells derived from the spleen of raw milk-treated mice, was not observed in the MLN. No significant differences were found between raw milk and shop milk in histone acetylation levels at Th1, Th17, and Treg loci (**Figure 5D**). After the challenges, histone acetylation of T-bet was increased in shop milk-treated mice when compared to raw milk-treated mice (**Figure 5E**), which resulted in a shift in the raw milk/shop milk ratio towards more favorable in shop milk after challenge (**Figure 5F**). A similar shift was observed for IL-10 (**Figure 5F**). Histone H3 acetylation was also assessed for MLN, but no significant differences between the groups were observed (**Figure S2**).

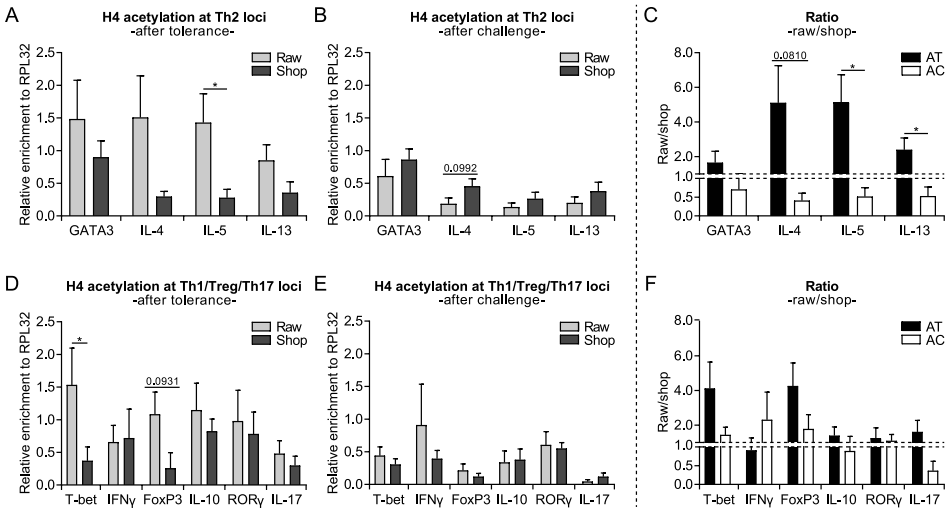


Figure 4. Increased histone acetylation of several T cell subset genes directly after raw milk exposure, while only Th2-related gene acetylation was reduced in raw milk-treated mice after both challenges. (**A**) Histone H4 acetylation at Th2 loci after the tolerance induction period (day -1), (**B**) after both challenges (day 34), and (**C**) the raw milk/shop milk ratio. (**D**) Histone H4 acetylation at Th1/Treg/Th17 loci after the tolerance induction period (day -1), (**E**) after both challenges (day 34), and (**F**) the raw milk/shop milk ratio. Histone H4 acetylation status was determined by means of chromatin immunoprecipitation in CD4⁺ T cells derived from splenocytes of raw milk- and shop milk-treated mice. The results are expressed as relative enrichment after normalization to ribosomal protein L32 (RPL32) as mean ± SEM, n = 6/group. *P < 0.05 as analyzed with an unpaired two-tailed Student's t-test. A Mann-Whitney test was used for T-bet, IFNγ, FoxP3, RORγ (after tolerance), T-bet, IL-17 (after model), and T-bet, IFNγ, RORγ (ratio raw/shop) since data did not obtain normality. Raw, raw cow's milk; shop, shop milk; AT, after tolerance; AC, after challenge.

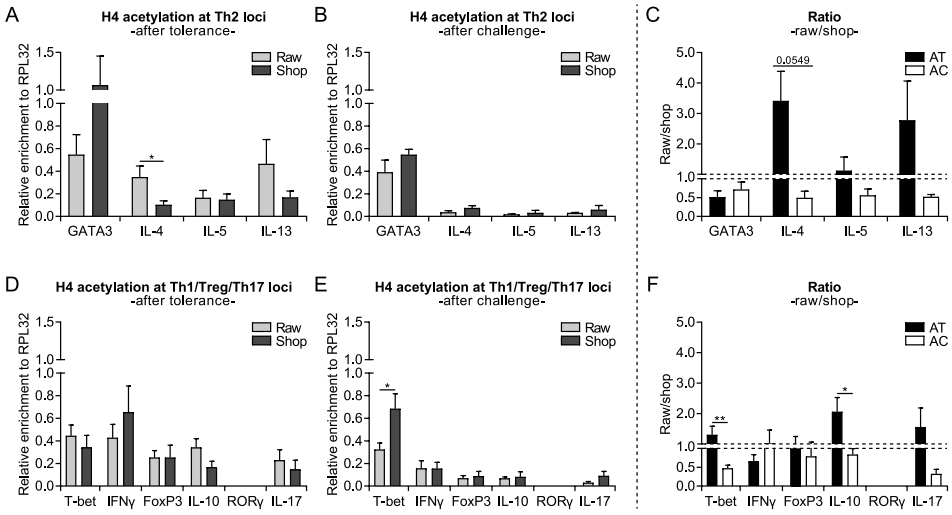


Figure 5. Raw milk-induced acetylation pattern of Th2-related genes observed in splenocyte-derived CD4⁺ T cells also visible locally in the mesenteric lymph nodes (MLN). (**A**) Histone H4 acetylation at Th2 loci after the tolerance induction period (day -1), (**B**) after both challenges (day 34), and (**C**) the raw milk/shop milk ratio. (**D**) Histone H4 acetylation at Th1/Treg/Th17 loci after the tolerance induction period (day -1), (**E**) after both challenges (day 34), and (**F**) the raw milk/shop milk ratio. Histone H4 acetylation status was determined by means of chromatin immunoprecipitation in MLN of raw milk- and shop milk-treated mice. The results are expressed as relative enrichment after normalization to ribosomal protein L32 (RPL32) as mean ± SEM, n = 4-6/group. *$P < 0.05$, **$P < 0.01$, as analyzed with an unpaired two-tailed Student's t-test. A Mann-Whitney test was used for GATA3, IL-10 (after tolerance), IL-10 (after model), and GATA3 (ratio raw/shop) since data did not obtain normality. Raw, raw cow's milk; shop, shop milk; AT, after tolerance; AC, after challenge.

Cytokine production by OVA-stimulated splenocytes corresponds to histone acetylation

Since differences in histone acetylation levels of cytokine genes do not necessarily result in differences in actual cytokine production, cytokine production upon *ex vivo* stimulation of splenocytes with OVA was measured. To be able to look at the OVA-specific cytokine response, the concentrations were only measured after both challenges (day 34). For the Th2-related cytokines IL-5 and IL-13, concentrations were low (**Figures 6A,B**). However, of interest is the tendency towards a reduced IL-5 production in raw milk-treated mice compared to shop milk-treated mice (**Figure 6A**), which corresponds to the lower IL-5 acetylation in splenocyte-derived CD4⁺ T cells that was observed in histones H4 and H3 (**Figures 4B and S1B**). IFNγ and IL-17 concentrations also correspond with the observed acetylation patterns, although no significant difference between the milk groups was observed (**Figures 4E and 6C,E**). In the case of IL-10, the cytokine concentration did not resemble gene acetylation, since the reduced IL-10 production in raw milk-treated mice was not observed in IL-10 gene acetylation (**Figures 4E and 6D**). *Ex vivo* stimulation of MLN with OVA did not result in measurable cytokine production (data not shown).

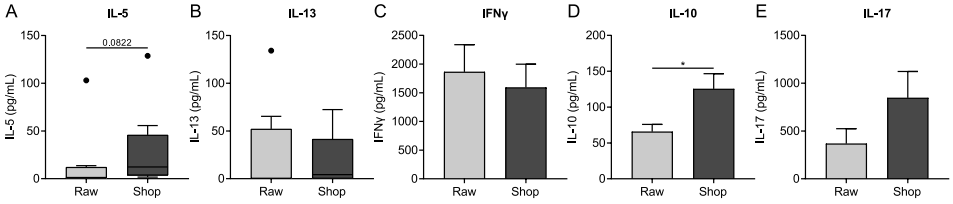


Figure 6. Cytokine concentrations produced by ovalbumin (OVA)-stimulated splenocytes corresponded with observed histone acetylation. (**A**) IL-5, (**B**) IL-13, (**C**), IFNγ, (**D**) IL-10, and (**E**) IL-17 concentrations measured in supernatant after *ex vivo* stimulation of splenocytes with OVA for four days (37 °C, 5% CO_2). Data are presented as box-and-whisker Tukey plot (in which outliers are shown as separately plotted points) for IL-5 and IL-13 concentrations and as mean ± SEM for IFNγ, IL-10, and IL-17 concentrations after subtracting baseline cytokine levels, n = 8/group. *P < 0.05 as analyzed with a Mann-Whitney test (**A, B**) or an unpaired two-tailed Student's t-test (**C-E**). Raw, raw cow's milk; shop, shop milk.

DISCUSSION

After showing causality in a murine house dust mite-induced asthma model (14), the present study demonstrates that raw, unprocessed, cow's milk is also protective in a murine model for food allergy. Raw milk induced oral tolerance to a non-milk, food allergen, by reducing acute allergic symptoms after intradermal challenge with OVA. This protective effect was not observed when a processed, shop milk was used to treat the mice. Looking at epigenetic modifications, raw milk exposure for eight days prior to sensitization led to higher histone acetylation of Th1-, Th2-, and Treg-related genes of splenocyte-derived $CD4^+$ T cells when compared to shop milk exposure. At the end of the study, after the induction of allergic symptoms, this general immune stimulation was resolved, and histone acetylation of Th2-related genes was lower when compared to shop milk. A similar, but less strong, pattern was locally visible, in the MLN. These results suggest that epigenetic regulation plays a role in the allergy-protective effect of raw milk.

Food allergies are thought to occur due to the failure to develop or the loss of oral tolerance (28). Oral tolerance is the phenomenon of local and systemic immune hyporesponsiveness to ingested food proteins (29). Actively inducing or restoring oral tolerance is an interesting approach for preventing or treating food allergies. For this, research has mainly focused on specific immunomodulation using the allergen. Both inducing oral tolerance by allergen exposure in early life and restoring oral tolerance via various types of allergen-specific immunotherapy are frequent topics of immunological research (30, 31). However, using the intact allergen for oral tolerance induction might also trigger sensitization or allergic symptoms in high-risk patients (32, 33).

Instead of specific immunomodulation, generic immunomodulation does not use the allergen to induce oral tolerance, preventing the risk of severe side effects. Generic immunomodulation is based on using beneficial immunomodulatory components that can create an environment that favors oral tolerance induction (34). Mainly dietary components, such as probiotics, prebiotics, synbiotics, and *n*-3 polyunsaturated fatty acids (PUFAs) have proven to be beneficial in this respect (35).

Several epidemiological studies already suggested that raw, unprocessed, cow's milk may have the capacity to prevent allergic diseases by inducing tolerance via generic immunomodulation. Raw cow's milk consumption was, for example, shown to be inversely associated with asthma, which indicated protection in the absence of the allergen (13). In a murine house dust mite-induced asthma model, we confirmed these findings by showing a causal relationship between raw cow's milk consumption and the prevention of allergic asthma (14). In the current study, raw cow's milk induced tolerance to OVA, an unrelated, non-milk, food allergen, which further substantiates this hypothesis.

Strikingly, processed, shop milk was not able to induce tolerance to OVA. This confirms earlier findings, which showed that milk processing abolishes the allergy-protective effect of raw milk (13-16). The milk processing chain consists of various steps to preserve milk along the supply chain. Each of these steps (e.g., machine milking, skimming, homogenization, heat treatment, storage, and packaging) induces changes in the composition of the milk, which makes it hard to pinpoint one particular raw milk constituent that is responsible for the protective effects (36). Even though comparing a raw milk with a shop milk (consumed by most people) was a logical first step in our opinion, future research should focus on testing milk from the same milk source that only differs in one processing step (skimmed milk, pasteurized milk, ultra-high temperature processing milk, etc.). Besides elucidating the raw milk component(s) involved, this will give the opportunity to look into the cellular mechanisms inducing tolerance in more depth.

Epigenetic regulation might be one of the mechanisms by which raw cow's milk exerts its allergy-protective effect. Since environmental factors are known to be able to modulate gene expression through epigenetic mechanisms, we wondered whether this also applied to raw milk. Epigenetic mechanisms can modify the accessibility of genes for transcription without altering the DNA nucleotide sequence, which means that they can modulate the phenotype without affecting the genotype (19). In this way, epigenetic mechanisms are key in the plasticity of gene expression. They are essential for developmental processes, like cellular differentiation, contributing, for example, to the flexibility among $CD4^+$ T cell subsets (37). The classical epigenetic mechanisms comprise DNA methylation and histone modifications, including histone acetylation, methylation, phosphorylation, and ubiquitination (20).

To determine the role of epigenetic mechanisms in the allergy-protective effect of raw milk, we assessed histone acetylation at the promoter regions of Th1-, Th2-, Th17-, and Treg-related genes of splenocyte-derived $CD4^+$ T cells and MLN. During histone acetylation, an acetyl group is added to a lysine residue at the N-terminal tail of a histone (mainly histones H3 and H4). This removes the positive charge on the histones that are involved, resulting in a decreased interaction with the negatively charged DNA. Consequently, the DNA is less tightly wrapped around the histones, which makes it more accessible to the transcriptional machinery. Therefore, higher histone acetylation usually results in higher gene transcription, while the opposite is true for reduced histone acetylation (19).

In line with the protective effects that were observed on acute allergic symptoms and IgE, histone acetylation of Th2-related genes (GATA3, IL-4, IL-5, and IL-13) of splenocyte-derived $CD4^+$ T cells after allergy induction was lower in raw milk-treated mice than in shop milk-treated mice. The strongest effects were observed on histone H4 acetylation at Th2 cytokine genes. Since histone acetylation substantially contributes to and is an important marker for an open chromatin structure (19, 20), we assessed whether the acetylation levels positively correlated with cytokine production. Unfortunately, Th2 cytokine concentrations were low, but the tendency towards a reduced IL-5 production in raw milk-treated mice as compared to shop milk-treated mice suggests that there is indeed a positive correlation. Several other studies already confirmed that differences in H4 acetylation levels at Th2 cytokine genes indeed correlate with cytokine production (26, 38). Since type 2 cytokines play a predominant role in allergic diseases by directing the effector phase of an allergic response (39), affecting epigenetic marks on Th2 cytokine genes might be an interesting preventive approach.

After allergy induction, the histone acetylation of Th1-, Th17-, and Treg-related genes did not differ between raw milk- and shop milk-treated mice. Although, here, histone acetylation patterns were reflected in cytokine production. The only cytokine for which the production did not correspond to gene acetylation was IL-10, suggesting that histone H3/H4 acetylation is not a main driver of IL-10 synthesis. Furthermore, we observed that IL-10 production was reduced in raw milk-treated mice as compared to shop milk-treated mice. This seems to be in contrast with the observed allergy protection, since IL-10 is known as a regulatory cytokine. However, in a murine model for OVA-induced food allergy, it was shown that IL-10 could also have proinflammatory effects. IL-10 was demonstrated to be essential for the development of food allergy by inducing mucosal mast cell expansion and activation (40). This indicates that lowering IL-10 concentrations in a murine OVA-induced food allergy model might be beneficial. Besides systemically looking at splenocyte-derived $CD4^+$ T cells, we also locally assessed histone acetylation in the MLN. Here, similar effects were observed, although less strong. This might have to

do with the fact that the whole tissue was used for ChIP analysis, rather than the isolated T cells. This may have resulted in weaker effects, as other cell types might also express the genes measured.

In addition to looking at histone acetylation patterns at the end of the study (after allergy induction), we also directly assessed histone acetylation after the eight days of milk exposure. Surprisingly, histone acetylation of the Th2-related genes of splenocyte-derived $CD4^+$ T cells was higher in the raw milk group as compared to the shop milk group. However, histone acetylation of T-bet and FoxP3 was also increased, suggesting a kind of general immune stimulation. Whether this general immune stimulation induced by raw milk is responsible for the observed allergy protection at the end of the study we do not know yet. Previously, however, it has been demonstrated that acquiring tolerance in food allergic children involves epigenetic regulation of the FoxP3 gene (41). Furthermore, epidemiological studies have shown that raw cow's milk consumption was associated with increased DNA demethylation of FoxP3 and increased numbers of Tregs (42). Unfortunately, we did not look at Treg numbers in our study, but since active suppression by Tregs is considered to be one of the main effector mechanisms for oral tolerance (43), the observed increase in histone acetylation of the FoxP3 gene might contribute to the allergy-protective effect. Inhibiting *de novo* histone acetylation with histone acetyltransferase inhibitors might be an interesting approach to further investigate the role of histone acetylation in the allergy-protective effect of raw milk.

How raw milk affects epigenetic marks on T cell-related genes is currently unclear, but there are some indications. Microbes derived from farm dust, known to prevent allergic asthma, were, for example, shown to operate via epigenetic mechanisms (44), suggesting that microbes that are present in raw milk might have similar effects. Furthermore, raw milk contains higher levels of *n*-3 PUFAs than industrially processed milk (15). These *n*-3 PUFAs reduce the risk of developing allergic diseases and they have been shown to lower the acetylation of IL-13 genes (45, 46). In addition, raw milk contains components, like lactoferrin, which can promote the growth of *Bifidobacteria* and *Lactobacilli* in the gut (17, 18). These bacteria are potent producers of short-chain fatty acids and these short-chain fatty acids are known for their capacity to inhibit histone deacetylases, thereby increasing gene transcription. Whether the above-mentioned components in the concentrations present in raw milk can influence epigenetic mechanisms and subsequently contribute to the allergy-protective effect of raw milk should be clarified in future studies. The possible involvement of epigenetic mechanisms should also be investigated in the case of the anti-allergic effects of human breast milk consumption (47).

In conclusion, we show the potency of raw cow's milk to induce tolerance to a non-milk, food allergen. This allergy-protective effect was abolished by industrial milk processing, emphasizing the importance of minimally processed milk. The allergy-protective constituents

of raw milk remain elusive and should be investigated in follow-up studies. In addition, we showed that raw milk is able to modulate gene expression through epigenetic mechanisms. By targeting histone marks on T cell-related genes, raw milk might have induced oral tolerance. Whether this is a cause-effect relationship and whether effects are more pronounced with longer raw milk exposure should be assessed in future research. Nevertheless, our data suggest that the consumption of certified raw cow's milk can contribute to allergy prevention and epigenetic regulations, especially histone modifications, might be one of the underlying mechanisms.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

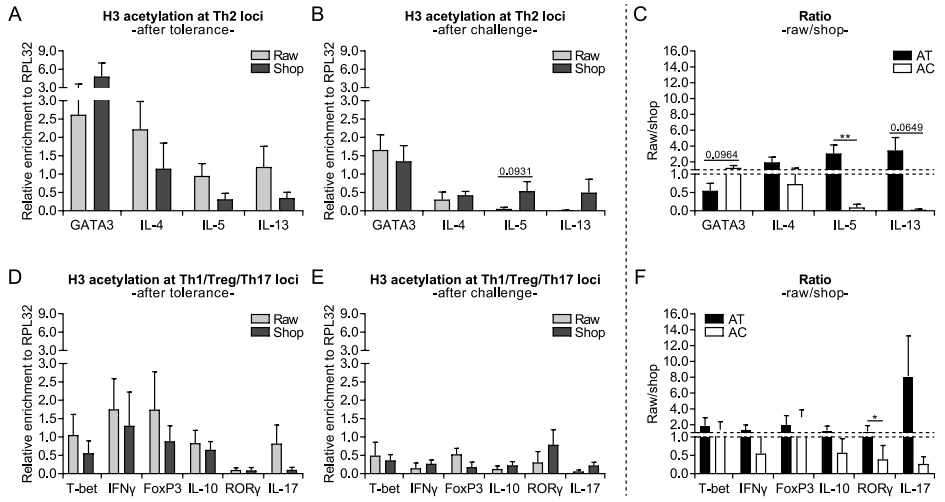


Figure S1. Acetylation patterns of histone H3 were comparable to histone H4 in splenocyte-derived CD4⁺ T cells. (**A**) Histone H3 acetylation at Th2 loci after the tolerance induction period (day -1), (**B**) after both challenges (day 34), and (**C**) the raw milk/shop milk ratio. (**D**) Histone H3 acetylation at Th1/Treg/Th17 loci after the tolerance induction period (day -1), (**E**) after both challenges (day 34), and (**F**) the raw milk/shop milk ratio. Histone H3 acetylation status was determined by means of chromatin immunoprecipitation in CD4⁺ T cells derived from splenocytes of raw milk- and shop milk-treated mice. Results are expressed as relative enrichment after normalization to ribosomal protein L32 (RPL32) as mean ± SEM, n = 6/group. *P < 0.05, **P < 0.01, as analyzed with a Mann-Whitney test. An unpaired two-tailed Student's t-test was used for IL-4, IL-5, IL-13, IL-10 (after tolerance), GATA3 (after model), and GATA3 (ratio raw/shop) since data obtained normality. Raw, raw cow's milk; shop, shop milk; AT, after tolerance; AC, after challenge.

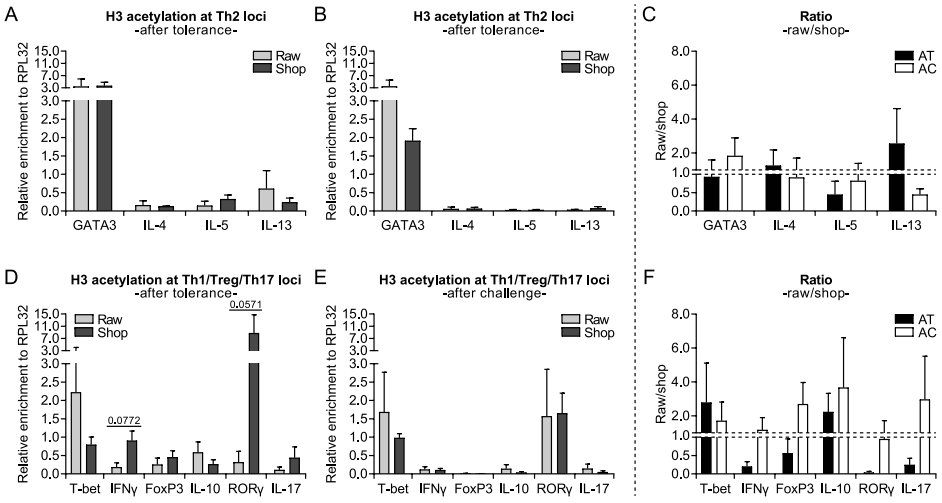


Figure S2. No differences between groups observed for histone H3 acetylation in mesenteric lymph nodes (MLN). (**A**) Histone H3 acetylation at Th2 loci after the tolerance induction period (day -1), (**B**) after both challenges (day 34), and (**C**) the raw milk/shop milk ratio. (**D**) Histone H3 acetylation at Th1/Treg/Th17 loci after the tolerance induction period (day -1), (**E**) after both challenges (day 34), and (**F**) the raw milk/shop milk ratio. Histone H3 acetylation status was determined by means of chromatin immunoprecipitation in MLN of raw milk- and shop milk-treated mice. Results are expressed as relative enrichment after normalization to ribosomal protein L32 (RPL32) as mean ± SEM, *n* = 3-6/group. No significant differences were observed. Raw, raw cow's milk; shop, shop milk; AT, after tolerance; AC, after challenge.

Table S1. Buffers used for chromatin immunoprecipitation (ChIP).

Lysis buffer I	**For 50 mL**
5 mM PIPES pH 8	0.5 mL 0.5 M PIPES pH 8
85 mM KCl	1.4 mL 3 M KCl
0.5% NP40 (Igepal-CA630)	0.25 mL Igepal (100%)
Protease inhibitor cocktail tablets	One tablet
Lysis buffer II	**For 50 mL**
10 mM Tris-HCl pH 7.5	0.5 mL 1 M Tris-HCl pH 7.5
150 mM NaCl	1.5 mL 5 M NaCl
1% NP40 (Igepal-CA630)	0.5 mL Igepal (100%)
1% DOC (Natriumdeoxycholat)	0.5 g
0.1% SDS	0.25 mL 20% SDS
1 mM EDTA	0.1 mL 0.5 M EDTA pH 8
Protease inhibitor cocktail tablets	One tablet
Wash buffer I	**For 50 mL**
20 mM Tris-HCl pH 8	1 mL Tris-HCl pH 8
150 mM NaCl	1.5 mL 5 M NaCl
2 mM EDTA	0.2 mL 0.5 M EDTA pH 8
0.1% SDS	0.25 mL 20% SDS
1% Triton X100	0.5 mL Triton X100
Wash buffer II	**For 50 mL**
20 mM Tris-HCl pH 8	1 mL Tris-HCl pH 8
500 mM NaCl	5 mL 5 M NaCl
2 mM EDTA	0.2 mL 0.5 M EDTA pH 8
0.1% SDS	0.25 mL 20% SDS
1% Triton X100	0.5 mL Triton X100
Wash buffer III	**For 50 mL**
10 mM Tris-HCl pH 8	0.5 mL Tris-HCl pH 8
1% IGEPAL CA630	0.5 mL IGEPAL (100%)
1% DOC (Sodium deoxycholat)	0.5 g
1 mM EDTA	0.1 mL 0.5 M EDTA pH 8
0.25 M LiCl	1.25 mL 10 M LiCl
1 x TE	**For 50 mL**
10 mM Tris-HCl pH 8	0.5 mL Tris-HCl pH 8
1 mM EDTA	0.1 mL 0.5 M EDTA pH 8
Elution buffer	**For 25 mL**
1% SDS	1.25 mL 20% SDS
0.1 M $NaHCO_3$	2.5 mL 1 M $NaHCO_3$

Table S2. Primers used for qPCR.

Locus	Sequence forward	Sequence reverse
RPL32	TCA TTT CTC AGG CAC ATC TT	ACT CAC CGT AAA ACA GAT GG
IL-4	TCT GCC TCC ATC ATC CTT CT	ACA CCA TAA TCG GCC TTT CA
IL-5	ACC CTG AGT TTC AGG ACT CG	TCC CCA AGC AAT TTA TTC TCT C
IL-10	CGA CCA GTT CTT TAG CGC TT	TGT GGC TTT GGT AGT GCA AG
IL-13	CAA CAA AGC AGA GAC CAG GG	CAG AGC CAG TGA GAG AAC CA
IL-17	TGG TTC TGT GCT GAC CTC AT	GCT CTC CCT GGA CTC ATG TT
GATA3	CAC TCG GAT TCC TCT CTC CC	CCA GGA GAG GGG TCG TTT AA
T-bet	CAC TGG TCC ACT GCT CTC TC	GAG ATG TCC GGT GGT GTC TC
RORγ	TGG GGT GCC TGT CAT CAT AC	TGA GAA CTT GGC TCC CTG TC
FoxP3	GAC TCA AGG GGG TCT CA	TTG GGC TTC ATC GGC AA
IFNγ	CAT ACC CTT TCC TTG CTT TTC	TTG TGG GAT TCT CTG AAA GCA

All oligonucleotides were synthesized by Metabion (Planegg, Germany).

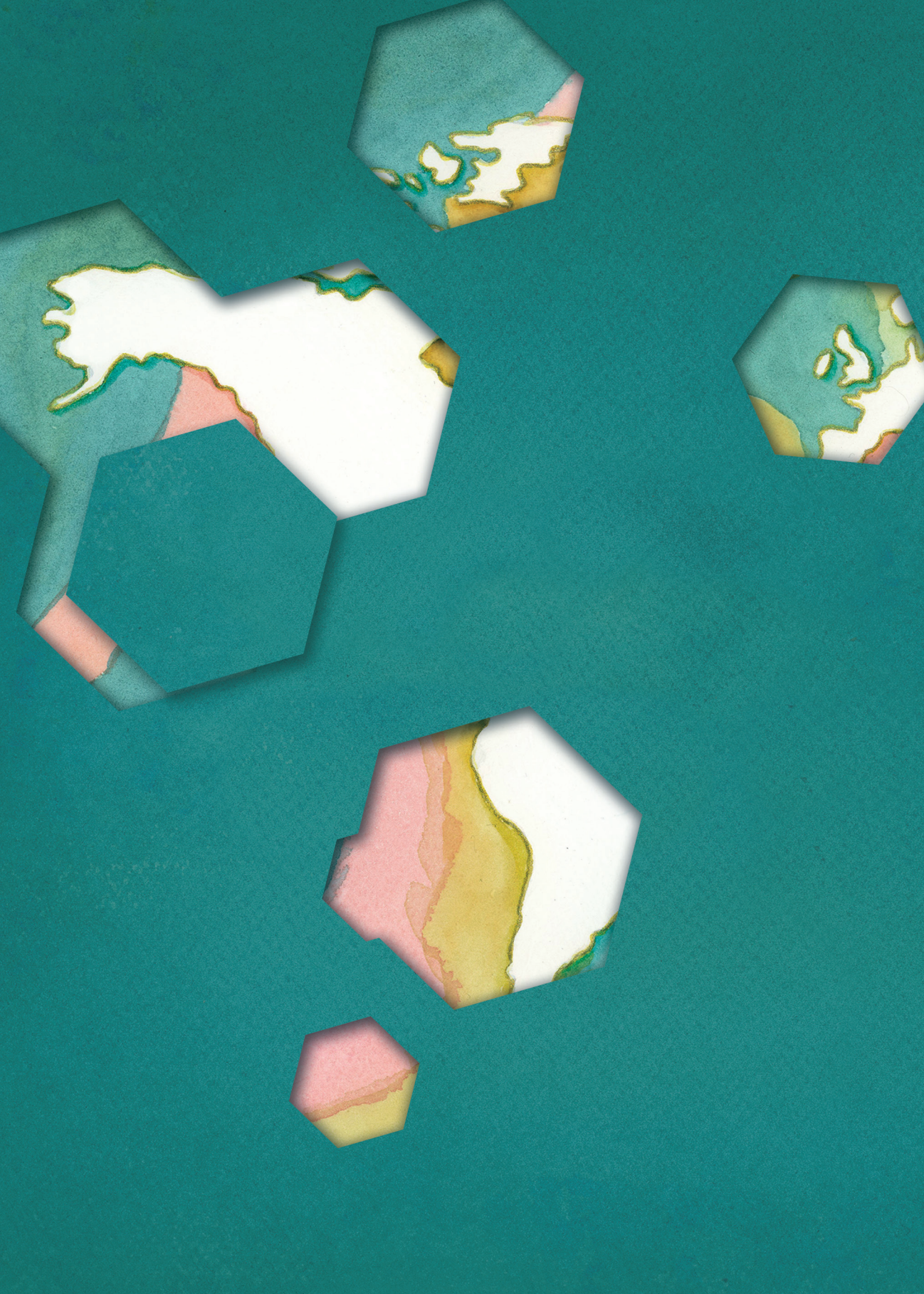
REFERENCES

1. Pawankar R, Canonica G, Holgate S, Lockey R, and Blaiss M. World Allergy Organisation (WAO) White book on allergy: update 2013. (2013) Available from: https://www.worldallergy.org/UserFiles/file/WhiteBook2-2013-v8.pdf Accessed on: 4 February 2019
2. Zuberbier T, Lotvall J, Simoens S, Subramanian SV, and Church MK. Economic burden of inadequate management of allergic diseases in the European Union: a GA(2)LEN review. *Allergy* (2014) 69(10): 1275-9.
3. Braun-Fahrlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, *et al*. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. *Clin Exp Allergy* (1999) 29(1): 28-34.
4. Kilpelainen M, Terho EO, Helenius H, and Koskenvuo M. Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* (2000) 30(2): 201-8.
5. von Ehrenstein OS, von Mutius E, Illi S, Baumann L, Bohm O, and von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* (2000) 30(2): 187-93.
6. Riedler J, Eder W, Oberfeld G, and Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin Exp Allergy* (2000) 30(2): 194-200.
7. Alfven T, Braun-Fahrlander C, Brunekreef B, von Mutius E, Riedler J, Scheynius A, *et al*. Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle - The PARSIFAL study. *Allergy* (2006) 61(4): 414-21.
8. von Mutius E and Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* (2010) 10(12): 861-8.
9. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
10. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, *et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
11. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
12. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
13. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
14. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T, *et al*. Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model. *Front Immunol* (2017) 8: 1045.
15. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-706 e13.
16. Brick T, Ege M, Boeren S, Bock A, von Mutius E, Vervoort J, *et al*. Effect of processing intensity on immunologically active bovine milk serum proteins. *Nutrients* (2017) 9(9): 963.

17. Abbring S, Hols G, Garssen J, and van Esch BCAM. Raw cow's milk consumption and allergic diseases - The potential role of bioactive whey proteins. *Eur J Pharmacol* (2019) 843: 55-65.
18. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
19. Potaczek DP, Harb H, Michel S, Alaskhar Alhamwe B, Renz H, and Tost J. Epigenetics and allergy: from basic mechanisms to clinical applications. *Epigenomics* (2017) 9(4): 539-71.
20. Alaskhar Alhamwe B, Khalaila R, Wolf J, von Bulow V, Harb H, Alhamdan F*, et al*. Histone modifications and their role in epigenetics of atopy and allergic diseases. *Allergy Asthma Clin Immunol* (2018) 14: 39.
21. Acevedo N, Frumento P, Harb H, Alashkar Alhamwe B, Johansson C, Eick L*, et al*. Histone acetylation of immune regulatory genes in human placenta in association with maternal intake of olive oil and fish consumption. *Int J Mol Sci* (2019) 20(5): 1060.
22. Bundesministerium der Justiz und für Verbraucherschutz. Verordnung über die Güteprüfung und Bezahlung der Anlieferungsmilch (Milch-Güteverordnung). (1980) Available from: http://www.gesetze-im-internet.de/milchg_v/index.html Accessed on: January 9 2019
23. Li XM, Schofield BH, Huang CK, Kleiner GI, and Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol* (1999) 103(2 Pt 1): 206-14.
24. Abbring S, Ryan JT, Diks MAP, Hols G, Garssen J, and van Esch BCAM. Suppression of food allergic symptoms by raw cow's milk in mice is retained after skimming but abolished after heating the milk - A promising contribution of alkaline phosphatase. *Nutrients* (2019) 11(7): 1499.
25. Abbring S, Kusche D, Roos TC, Diks MAP, Hols G, Garssen J*, et al*. 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(7): 1013-25.
26. Harb H, Amarasekera M, Ashley S, Tulic MK, Pfefferle PI, Potaczek DP*, et al*. Epigenetic regulation in early childhood: a miniaturized and validated method to assess histone acetylation. *Int Arch Allergy Immunol* (2015) 168(3): 173-81.
27. Haring M, Offermann S, Danker T, Horst I, Peterhansel C, and Stam M. Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. *Plant Methods* (2007) 3: 11.
28. Chinthrajah RS, Hernandez JD, Boyd SD, Galli SJ, and Nadeau KC. Molecular and cellular mechanisms of food allergy and food tolerance. *J Allergy Clin Immunol* (2016) 137(4): 984-97.
29. Pabst O and Mowat AM. Oral tolerance to food protein. *Mucosal Immunol* (2012) 5(3): 232-9.
30. Palmer DJ and Prescott SL. Does early feeding promote development of oral tolerance? *Curr Allergy Asthma Rep* (2012) 12(4): 321-31.
31. Kostadinova AI, Willemsen LE, Knippels LM, and Garssen J. Immunotherapy - Risk/benefit in food allergy. *Pediatr Allergy Immunol* (2013) 24(7): 633-44.
32. Bellach J, Schwarz V, Ahrens B, Trendelenburg V, Aksunger O, Kalb B*, et al*. Randomized placebo-controlled trial of hen's egg consumption for primary prevention in infants. *J Allergy Clin Immunol* (2017) 139(5): 1591-9 e2.
33. Palmer DJ, Metcalfe J, Makrides M, Gold MS, Quinn P, West CE*, et al*. Early regular egg exposure in infants with eczema: a randomized controlled trial. *J Allergy Clin Immunol* (2013) 132(2): 387-92 e1.
34. Gourbeyre P, Denery S, and Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. *J Leukoc Biol* (2011) 89(5): 685-95.
35. Vonk MM, Kostadinova AI, Kopp MV, van Esch BCAM, Willemsen LEM, Knippels LMJ*, et al*.

16 - Dietary interventions in infancy in *Allergy, Immunity and Tolerance in Early Childhood*, U. Wahn and H.A. Sampson, Editors. (2016); 261-84.

36. Michalski MC and Januel C. Does homogenization affect the human health properties of cow's milk? *Trends Food Sci Tech* (2006) 17(8): 423-37.
37. Suarez-Alvarez B, Rodriguez RM, Fraga MF, and Lopez-Larrea C. DNA methylation: a promising landscape for immune system-related diseases. *Trends Genet* (2012) 28(10): 506-14.
38. Harb H, Raedler D, Ballenberger N, Bock A, Kesper DA, Renz H, *et al*. Childhood allergic asthma is associated with increased IL-13 and FOXP3 histone acetylation. *J Allergy Clin Immunol* (2015) 136(1): 200-2.
39. Gandhi NA, Bennett BL, Graham NM, Pirozzi G, Stahl N, and Yancopoulos GD. Targeting key proximal drivers of type 2 inflammation in disease. *Nat Rev Drug Discov* (2016) 15(1): 35-50.
40. Polukort SH, Rovatti J, Carlson L, Thompson C, Ser-Dolansky J, Kinney SR, *et al*. IL-10 enhances IgE-mediated mast cell responses and is essential for the development of experimental food allergy in IL-10-deficient mice. *J Immunol* (2016) 196(12): 4865-76.
41. Paparo L, Nocerino R, Cosenza L, Aitoro R, D'Argenio V, Del Monaco V, *et al*. Epigenetic features of FoxP3 in children with cow's milk allergy. *Clin Epigenetics* (2016) 8: 86.
42. Lluis A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D, *et al*. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol* (2014) 133(2): 551-9.
43. Weiner HL. Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes Infect* (2001) 3(11): 947-54.
44. Brand S, Teich R, Dicke T, Harb H, Yildirim AO, Tost J, *et al*. Epigenetic regulation in murine offspring as a novel mechanism for transmaternal asthma protection induced by microbes. *J Allergy Clin Immunol* (2011) 128(3): 618-25 e1-7.
45. D'Vaz N, Meldrum SJ, Dunstan JA, Lee-Pullen TF, Metcalfe J, Holt BJ, *et al*. Fish oil supplementation in early infancy modulates developing infant immune responses. *Clin Exp Allergy* (2012) 42(8): 1206-16.
46. Harb H, Irvine J, Amarasekera M, Hii CS, Kesper DA, Ma Y, *et al*. The role of PKCzeta in cord blood T-cell maturation towards Th1 cytokine profile and its epigenetic regulation by fish oil. *Biosci Rep* (2017) 37(2): BSR20160485.
47. Rajani PS, Seppo AE, and Jarvinen KM. Immunologically active components in human milk and development of atopic disease, with emphasis on food allergy, in the pediatric population. *Front Pediatr* (2018) 6: 218.



CHAPTER 5

Suppression of food allergic symptoms by raw cow's milk in mice is retained after skimming but abolished after heating the milk – A promising contribution of alkaline phosphatase

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ABSTRACT

Raw cow's milk was previously shown to suppress allergic symptoms in a murine model for food allergy. In the present study, we investigated the contribution of fat content and heat-sensitive milk components to this allergy-protective effect. In addition, we determined the potency of alkaline phosphatase (ALP), a heat-sensitive raw milk component, to affect the allergic response. C3H/HeOuJ mice were treated with raw milk, pasteurized milk, skimmed raw milk, pasteurized milk spiked with ALP, or PBS for eight days prior to sensitization and challenge with ovalbumin (OVA). Effects of these milk types on the allergic response were subsequently assessed. Similar to raw milk, skimmed raw milk suppressed food allergic symptoms, demonstrated by a reduced acute allergic skin response and low levels of OVA-specific IgE and Th2-related cytokines. This protective effect was accompanied by an induction of $CD103^{+}CD11b^{+}$ dendritic cells and TGF-β-producing regulatory T cells in the mesenteric lymph nodes. Pasteurized milk was not protective but adding ALP restored the allergy-protective effect. Not the fat content, but the heat-sensitive components are responsible for the allergy-protective effects of raw cow's milk. Adding ALP to heat-treated milk might be an interesting alternative to raw cow's milk consumption, as spiking pasteurized milk with ALP restored the protective effects.

INTRODUCTION

Breastfeeding is the gold standard of infant nutrition. It is a complex matrix providing a unique combination of lipids, carbohydrates, proteins, vitamins, and minerals. In addition, breast milk contains numerous components with immunomodulatory properties, such as immunoglobulins, lactoferrin, oligosaccharides, long-chain fatty acids, antioxidants, and anti-inflammatory cytokines (1). These bioactive components are potentially responsible for the allergy-protective effects associated with breastfeeding (2-4).

In analogy to breast milk, numerous epidemiological studies have shown that the consumption of raw, unprocessed, cow's milk can also reduce the risk of allergic diseases (5-9). These epidemiological findings were recently confirmed by causal evidence, showing that raw cow's milk prevents the development of house dust mite-induced allergic asthma (10) and of ovalbumin (OVA)-induced food allergy (11) in murine animal models. However, due to the possible contamination with pathogens, raw cow's milk consumption is discouraged by regulatory authorities (12). Even though risks from certified raw cow's milk, produced under strict hygienic and microbiological standards, are considered to be low (13), a zero-risk can never be attained. Cow's milk used for commercial purposes is therefore processed.

Milk processing, i.e., heat treatment and homogenization, ensures microbial safety and increases shelf life. Unfortunately, it also impacts the asthma- and allergy-protective effect of raw cow's milk (5, 10, 11, 14). Milk processing considerably alters raw cow's milk with most prominent effects on the fat content and heat-sensitive milk components. For both constituents, associations have been found in relation to the asthma- and allergy-protective effects. For the fat content of the milk, effects were mainly attributed to the levels of *n*-3 polyunsaturated fatty acids (14), whereas for the heat-sensitive milk components, the whey protein fraction was found to be associated with a reduced allergy risk (5). Confirming that these raw milk constituents are indeed responsible for the observed allergy protection by showing causality is crucial. This knowledge will further support the development of mildly processed milk, or the addition of specific raw milk ingredients to heat-treated milk as an alternative to raw milk consumption.

In the current study, we investigated to which extent the fat content of the milk and the heat-sensitive milk components contribute to the allergy-protective effects of raw cow's milk by examining skimmed raw milk and pasteurized milk, respectively, in a murine OVA-induced food allergy model. In addition, we added alkaline phosphatase (ALP), one of the first bioactive raw milk components losing its activity upon heat treatment, to pasteurized milk to assess whether this restores the allergy-protective effect.

MATERIALS & METHODS

Mice

Three-week-old, specific pathogen-free, female C3H/HeOuJ mice, purchased from Charles River Laboratories (Sulzfeld, Germany) were housed in filter-topped makrolon cages (one cage/group, *n* = 6-8/cage) at the animal facility of Utrecht University (Utrecht, The Netherlands) on a 12 h light/dark cycle with unlimited access to food ('Rat and Mouse Breeder and Grower Expanded'; Special Diet Services, Witham, UK) and water. Upon arrival, mice were randomly allocated to the control or experimental groups and were habituated to the laboratory conditions for one week prior to the start of the study. Animal procedures were approved by the Ethical Committee for Animal Research of the Utrecht University and conducted according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes (AVD108002015346).

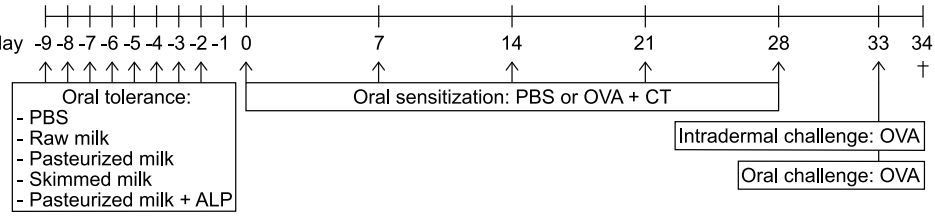
Milk types

Raw cow's milk was collected from a dairy farm (Macroom, Ireland). After collection, the raw cow's milk was divided into three aliquots. Aliquot 1 was stored without any treatment at -20 °C until further use (raw milk). Aliquot 2 was heated for 15 s at 78 °C, cooled to 4 °C and then stored at -20 °C until further use (pasteurized milk). Aliquot 3 was skimmed at 55 °C to remove the milk fat, cooled to 4 °C and stored at -20 °C until further use (skimmed milk; 0.1% fat). All milk types were produced for experimental purposes only (Danone Nutricia Research, Utrecht, The Netherlands). On the days of milk treatment (experimental days -9 to -2; **Figure 1**), milks were thawed at room temperature and part of the pasteurized milk was spiked with bovine intestinal ALP (pasteurized milk + ALP; 3 units/0.5 mL pasteurized milk; 10× higher concentration than present in raw cow's milk). ALP was kindly provided by prof. dr. W. Seinen (Alloksys Life Sciences BV/AMRIF BV, Wageningen, The Netherlands).

Animal procedures

A schematic representation of the experimental design is shown in **Figure 1**. On experimental days 0, 7, 14, 21, and 28, mice (*n* = 8/group) were orally sensitized to 20 mg of the hen's egg protein OVA (grade V; Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in 0.5 mL PBS containing 10 µg cholera toxin (CT; List Biological Laboratories, Campbell, CA, USA) as an adjuvant. The PBS-sensitized control mice (*n* = 6) received CT alone (10 µg/0.5 mL PBS). Prior to sensitization, mice were orally treated by using a blunt needle with 0.5 mL raw milk, pasteurized milk, skimmed raw milk, pasteurized milk spiked with ALP, or PBS (as a control) for eight consecutive days (days -9 to -2). On day 27, one day before the last sensitization, a blood sample was drawn via cheek puncture to measure basophil activation. On day 33, five days after the last sensitization, all mice were challenged intradermally in both ears with OVA (10 µg/20 µL PBS) to determine the

acute allergic skin response. On the same day, mice were challenged orally with 50 mg OVA dissolved in 0.5 mL PBS. Sixteen hours after the oral challenge (day 34), a blood sample was taken, and mice were killed by cervical dislocation.



Group	*n*	Tolerance	Sensitization	Challenge
PBS	6	PBS	PBS + CT	OVA
OVA	8	PBS	OVA + CT	OVA
Raw milk	8	Raw milk	OVA + CT	OVA
Pasteurized milk	8	Pasteurized milk	OVA + CT	OVA
Skimmed milk	8	Skimmed milk	OVA + CT	OVA
Pasteurized milk + ALP	8	Pasteurized milk + ALP	OVA + CT	OVA

Figure 1. Schematic overview of the experimental setup. Female C3H/HeOuJ mice were randomly allocated to the control or experimental groups: PBS group (PBS-sensitized control mice; *n* = 6), OVA group (OVA-sensitized allergic mice; *n* = 8), raw milk group (raw milk-treated mice; *n* = 8), pasteurized milk group (pasteurized milk-treated mice; *n* = 8), skimmed milk group (skimmed milk-treated mice; *n* = 8) and pasteurized milk + ALP group (pasteurized milk + ALP-treated mice; *n* = 8). Mice were orally treated with 0.5 mL raw milk, pasteurized milk, skimmed milk, pasteurized milk spiked with ALP, or PBS (as a control). Following this oral tolerance induction period, mice were orally sensitized to OVA (20 mg/0.5 mL PBS) with CT as an adjuvant (10 µg/0.5 mL). PBS-sensitized control mice (PBS group) received CT alone. Subsequently, all mice were intradermally and orally challenged with OVA. Mice were killed on day 34 (as indicated by †). ALP, alkaline phosphatase; OVA, ovalbumin; CT, cholera toxin.

Evaluation of the acute allergic skin response

To assess the magnitude of the acute allergic skin response to OVA, mice were intradermally challenged in the ear pinnae of both ears with 10 µg OVA in 20 µL PBS. Ear thickness was measured in duplicate for each ear prior to and 1 h after the intradermal challenge using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). By subtracting the mean basal ear thickness from the mean ear thickness measured 1 h after the intradermal challenge, the ear swelling (expressed as Δ µm) was calculated. Isoflurane (Abbott, Breda, The Netherlands) was used for inhalation anesthesia to perform the intradermal challenge as well as the ear measurements. Measurements were performed blinded.

Basophil activation test

The basophil activation test was performed as described previously (15), with few alterations. Briefly, whole blood was drawn from each mouse via cheek puncture on experimental day 27 (one day before the last sensitization). Blood samples from two mice were pooled and

incubated with RPMI 1640 medium (Lonza, Verviers, Belgium), anti-mouse IgE (0.125 µg/mL; eBioscience, Breda, The Netherlands), or OVA (20 µg/mL; Sigma-Aldrich) for 90 min at 37 °C. Activation was stopped with PBS containing 5 mM EDTA (Thermo Fisher Scientific, Paisley, Scotland). After washing the cells twice with PBS, red blood cells were lysed and samples were fixed using a whole blood lysing reagent kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. Cells were then washed again, and non-specific binding sites were blocked by incubating cells for 15 min on ice with anti-mouse CD16/CD32 (Mouse BD Fc Block; BD Biosciences, Alphen aan de Rijn, The Netherlands). Cells were subsequently stained for 30 min on ice with CD4-PE and CD45R/B220-PE to gate out T cells and B cells and with IgE-FITC and CD49b-APC to select basophils. CD200R-PerCP-eFluor® 710 was used as a marker for basophil activation. All antibodies were purchased from eBioscience. Flow cytometry was performed using FACS Canto II (BD Biosciences) and the results were analyzed using FlowLogic Software (Inivai Technologies, Mentone, Australia). Cut-off gates for positivity were established using the fluorescence-minus-one technique.

Measurement of OVA-specific immunoglobulins in serum

Blood samples collected prior to sacrifice were centrifuged at 10,000 rpm for 10 min and serum was stored at -20 °C until analysis of OVA-specific immunoglobulins by means of ELISA. OVA-specific IgE levels were quantified as described previously (16), with few modifications. Briefly, high binding Costar 9018 plates (Corning Inc., New York, NY, USA) were coated overnight at 4 °C with 2 µg/mL purified rat anti-mouse IgE (BD Biosciences) in carbonate/bicarbonate buffer (0.05 M, pH 9.6; Sigma-Aldrich). The next day, plates were washed, blocked for 1 h with PBS/1% bovine serum albumin (BSA; Sigma-Aldrich) and incubated for 2 h with serum samples at room temperature. After washing, plates were incubated for 1 h with 1 µg/mL OVA coupled to digoxigenin (DIG). Plates were then washed again, followed by 1 h incubation with 300 mU/mL anti-DIG-POD Fab fragments conjugated to horseradish peroxidase (Sigma-Aldrich). After washing again, the reaction was developed using *o*-phenylenediamine (Sigma-Aldrich) and stopped by 4 M H_2SO_4. The absorbance was measured at 490 nm using a Benchmark microplate reader (Bio-Rad, Veenendaal, The Netherlands). OVA-specific IgE levels are expressed in arbitrary units, calculated based on a titration curve of pooled sera serving as an internal standard. For OVA-specific IgG1 and IgA, high binding Costar 9018 plates were coated with 20 µg/mL OVA (Sigma-Aldrich) in carbonate/bicarbonate buffer and incubated overnight at 4 °C. After overnight incubation, plates were washed and blocked for 1 h with PBS/1% BSA. Serum samples were then incubated for 2 h at room temperature and after washing, plates were incubated for 1.5 h with biotinylated rat anti-mouse IgG1 or IgA detection antibody (1 µg/mL; BD Biosciences). Plates were subsequently washed, incubated for 45 min with streptavidin-horseradish peroxidase (0.5 µg/mL; Sanquin, Amsterdam, The Netherlands), washed again and developed as described above for IgE. OVA-specific IgG1 and IgA levels are expressed as optical density (OD) values.

Spleen, mesenteric lymph nodes (MLN) and lamina propria (LP) cell isolation

Spleen and MLN single cell suspensions were obtained by crushing tissues through a 70 µm nylon cell strainer using a syringe. Splenocyte suspensions were incubated with lysis buffer (8.3 g NH_4Cl, 1 g KHC_3O, and 37.2 mg EDTA dissolved in 1 L demi water, filter sterilized) to remove red blood cells. Cell suspensions were resuspended in RPMI 1640 medium (Lonza), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands), penicillin (100 U/mL)/streptomycin (100 µg/mL; Sigma-Aldrich) and β-marcaptoethanol (20 µM; Thermo Fisher Scientific) prior to *ex vivo* OVA-specific restimulation assays or in PBS/1% BSA (Sigma-Aldrich) prior to cell stainings for flow cytometric analysis. For the isolation of small intestinal LP cells (n = 6/group), the small intestine was removed, cleared from fat and Peyer's patches, opened longitudinally, washed in PBS, and cut into 0.5 cm pieces. To remove epithelial cells and intraepithelial lymphocytes, these pieces were washed using Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific) containing 15 mM HEPES (Thermo Fisher Scientific), pH 7.2, and incubated 4 × 15 min at 37 °C with HBSS/HEPES buffer supplemented with 5 mM EDTA, 10% FBS and penicillin (100 U/mL)/streptomycin (100 µg/mL), pH 7.2. After washing with RPMI 1640 medium containing 5% FBS and penicillin/streptomycin, tissue samples were digested for 2 × 45 min on a plate shaker at 37 °C with RPMI 1640 medium supplemented with 5% FBS, penicillin/streptomycin and 0.5 mg/mL collagenase type VIII (Sigma-Aldrich). To collect lamina propria cells, samples were vortexed for 10 s after each incubation and passed through a 100 µm nylon cell strainer. LP cell suspensions were subsequently washed with HBSS/HEPES and purified using a Percoll® density gradient (pH 7.2; GE Healthcare, Uppsala, Sweden). Purified LP cell suspensions were washed and resuspended in PBS/1% BSA for flow cytometric analysis.

Flow cytometric analysis of immune cells

Spleen-, MLN-, and LP-derived single cell suspensions (0.5-1 × 10^6 cells/well) were incubated for 15 min on ice with anti-mouse CD16/CD32 (Mouse BD Fc Block; BD Biosciences) in PBS/1% BSA/5% FBS buffer to block non-specific binding sites. Subsequently, cells were extracellularly stained with CD4-PerCP-Cy5.5, CD69-APC, CXCR3-PE, CD25-Alexa Fluor® 488, F4/80-APC-eFluor® 780, CD11c-PerCP-Cy5.5, CD103-APC, CD11b-PE, MHCII-FITC, CD45-PE-Cy7, CD19-PerCP-Cy5.5, CD45R/B220-FITC, latency-associated peptide (LAP)-PE-Cy7 (all purchased from eBioscience), T1ST2-FITC (MD Bioproducts, St. Paul, MN, USA) or CD138-APC (BD Biosciences) for 30 min on ice. Viable cells were distinguished using Fixable Viability Dye-eFluor® 780 (eBioscience). Cells only stained for extracellular markers were fixed using IC Fixation Buffer (eBioscience). Cells additionally stained with intracellular markers were fixed and permeabilized using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol and then stained with FoxP3-PE-Cy7 or -APC (eBioscience). Stained cells were measured on the FACS Canto II (BD Biosciences) and analyzed with FlowLogic Software (Inivai Technologies). To increase LAP expression on the

surface of MLN-derived lymphocytes, cells were polyclonally stimulated with anti-CD3 (10 µg/mL)/CD28 (1 µg/mL; eBioscience) for 48 h at 37 °C, 5% CO_2 prior to staining, and boosted afterwards with leukocyte activation cocktail (BD Biosciences) for 4 h at 37 °C, 5% CO_2.

Cytokine measurements after *ex vivo* OVA-specific stimulation of splenocytes

Single cell splenocyte suspensions (8×10^5 cells/well) were cultured in U-bottom culture plates (Greiner, Frickenhausen, Germany) with either medium or OVA (50 µg/mL) for four days at 37 °C, 5% CO_2. Culture supernatant was collected and stored at -20 °C until measurements of IFNγ, IL-13 and IL-10 by means of ELISA as described elsewhere (17).

Short-chain fatty acid (SCFA) analysis in caecum

Caecal content was collected, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. After thawing, samples were homogenized by vortexing and diluted in cold PBS (1:10). Samples were subsequently centrifuged (13,000 rpm, 10 min), the supernatant was collected and concentrations of acetic, propionic, butyric, isobutyric, valeric, and isovaleric acid were determined as previously described (18) by means of a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan), using 2-ethylbutyric acid as internal standard.

Statistical analysis

Data are presented as mean ± SEM and differences between pre-selected groups were statistically determined with one-way ANOVA followed by a Bonferroni's multiple comparisons test. For plasma cells in the MLN, log-transformed data were used to obtain normality for one-way ANOVA. For the same reason, OVA-specific IgG1 and IgA levels were square root-transformed. As OVA-specific IgE levels were not normally distributed, data were presented in a box-and-whisker Tukey plot and analyzed using Kruskal-Wallis test followed by a Dunn's multiple comparisons test for pre-selected groups. All statistical analyses were performed using GraphPad Prism software (version 7.03; GraphPad Software, San Diego, CA, USA) and results were considered statistically significant when $P < 0.05$.

RESULTS

Suppression of the allergic effector response by raw milk is retained after skimming but abolished after heating the milk

To determine whether milk processing affects the capacity of raw cow's milk to induce tolerance to a non-milk, food allergen, mice were orally treated with raw milk, pasteurized milk or skimmed milk before being sensitized and challenged with OVA. As expected, OVA-sensitized allergic mice showed an increased acute allergic skin response upon intradermal challenge compared to PBS-sensitized control mice (**Figure 2A**). Exposing mice to raw milk

before OVA sensitization significantly reduced the acute allergic skin response compared to PBS-treated allergic mice (**Figure 2A**). This protective effect was retained after skimming but abolished after pasteurization of the milk (**Figure 2A**). Since ALP is one of the first bioactive raw milk components losing activity upon heat treatment, we investigated whether spiking pasteurized milk with ALP would restore the allergy-protective effect. Interestingly, addition of ALP to pasteurized milk significantly lowered the acute allergic skin response compared to PBS-treated allergic mice and pasteurized milk-treated mice (**Figure 2A**). To study the extent of basophil activation, basophil surface expression of CD200R after stimulation of whole blood with OVA was determined. Even though no difference was observed in CD200R expression on basophils of OVA-sensitized allergic mice compared to PBS-sensitized control mice, CD200R expression was significantly reduced on basophils of mice treated with pasteurized milk + ALP compared to mice treated with pasteurized milk alone (**Figure 2B**), which is in line with the effects observed on the acute allergic skin response (**Figure 2A**). OVA-specific IgE levels and plasma cells were not significantly affected by exposure to the different milk types, but they did follow a similar pattern as the acute allergic skin response, with low levels in the raw milk, skimmed milk and pasteurized milk + ALP group and higher levels in the pasteurized milk group (**Figures 2C,D**). Unfortunately, OVA-specific IgE levels were not significantly increased in OVA-sensitized allergic mice compared to PBS-sensitized control mice (**Figure 2C**). However, OVA-specific IgG1 and IgA did (tend to) increase in these mice, demonstrating an immune response to OVA and supporting sensitization (**Figures 2E,F**). Functionality of IgE antibodies was furthermore confirmed using a murine bone marrow-derived mast cell degranulation assay (data not shown). For OVA-specific IgG1 and IgA, no differences between milk groups were observed (**Figures 2E,F**).

Low Th2-related cytokine production by splenocytes from raw milk- and skimmed milk-treated mice after *ex vivo* stimulation with OVA

To investigate whether different milk types affect T helper cell phenotype, spleen and MLN cells were isolated and analyzed by flow cytometry. Percentages of Th1 and Th2 cells were not affected in OVA-sensitized allergic mice compared to PBS-sensitized control mice (**Figures 3A-D**). However, in the spleen of mice treated with pasteurized milk, Th1 cells tended to decrease compared to allergic mice treated with PBS (**Figure 3A**). Th2 cell frequency in the spleen did not differ between milk groups (**Figure 3B**). In the MLN, the percentage of Th1 cells did not differ between milk groups, whereas Th2 cell frequency was increased in mice treated with pasteurized milk + ALP compared to allergic mice treated with PBS (**Figure 3D**). To determine the functional response of splenocytes and MLN cells upon exposure to OVA, cytokine production was determined. Th1-related IFNγ production by splenocytes was not affected by the different milk types (**Figure 3E**). For the Th2-related cytokine IL-13, low concentrations were observed in the raw milk, skimmed milk and pasteurized milk + ALP group (**Figure 3F**), which coincided with the effects observed on the acute allergic skin response

(**Figure 2A**). Pasteurized milk treatment tended to increase the IL-13 production compared to raw milk treatment, whereas adding ALP to pasteurized milk tended to restore the low IL-13 levels (**Figure 3F**). Compared to raw milk, pasteurized milk also increased the production of IL-10 (**Figure 3G**), which was previously shown to act as Th2 cytokine in this OVA-induced food allergy model (19). *Ex vivo* stimulation of MLN cells with OVA did not induce detectable cytokine production (data not shown).

Raw milk and skimmed milk induce tolerance-associated cell types in the MLN

To assess whether the prevention of OVA-induced food allergic symptoms by raw milk, skimmed milk and pasteurized milk + ALP was associated with the induction of tolerance-associated cell types, changes in different dendritic cell (DC) and regulatory T cell (Treg) subsets were determined in the MLN. DC ($CD11c^+MHCII^+$) numbers tended to increase in raw milk-treated mice and increased in skimmed milk-treated mice compared to PBS-treated allergic mice (**Figure 4A**). More specific assessment of the DC subsets affected, revealed that both milk types mainly increased the tolerogenic $CD103^+CD11b^+$ DC subpopulation (**Figure 4B**). Although $CD103^+$ DCs are known for their capacity to induce $FoxP3^+$ Tregs in the MLN (20), no differences between groups were observed in the percentage of $CD25^+FoxP3^+$ Treg cells (**Figure 4C**). However, interestingly, the Treg subtype secreting TGF-β, also known as Th3 cells, tended to increase in the raw milk group compared to the pasteurized milk group (**Figure 4D**).

Increased percentage of tolerogenic DCs in MLN of raw milk- and skimmed milk-treated mice is not associated with increased Treg cell frequency in the LP

Besides promoting the differentiation of naïve T cells into Treg cells, $CD103^+$ DCs also induce the expression of gut-homing receptors on the surface of Treg cells (20). To investigate whether the increased tolerogenic $CD103^+CD11b^+$ DC subpopulation in the MLN of raw milk- and skimmed milk-treated mice was associated with increased Treg cell trafficking to the gut, lamina propria cells were isolated and analyzed by flow cytometry. However, $CD25^+FoxP3^+$ Treg frequency did not differ between milk groups (**Figure 5C**) and also $CD11c^+MHCII^+$ DCs and the $CD103^+CD11b^+$ subset showed no differences in the LP (**Figures 5A,B**).

Different milk types did not affect SCFA concentrations

Since modulation of the gut microbiome might be a way in which raw milk, skimmed milk and pasteurized milk + ALP induced tolerance to OVA, metabolic activity of the gut microbiome was assessed by determining SCFA concentrations in the caecum of the mice. Total SCFA concentrations were not significantly different between groups, but skimmed milk- and pasteurized milk + ALP-treated mice showed the highest levels (**Figure 6A**). Regarding individual SCFA, a similar pattern was observed for butyric acid and acetic acid concentrations, although again differences did not reach significance (**Figures 6B,C**). For propionic acid, concentrations were comparable in each milk group (**Figure 6D**).

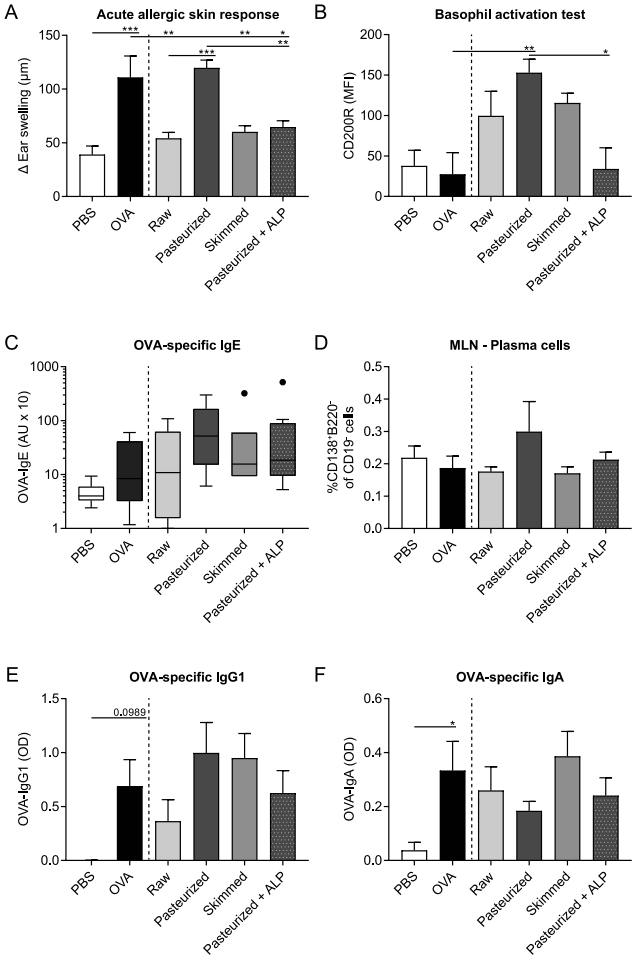


Figure 2. The protective effect of raw milk on the allergic effector response is retained by skimming but abolished by pasteurization of the milk. (**A**) The acute allergic skin response, expressed as Δ ear swelling, measured after intradermal challenge in the ear pinnae of both ears with OVA. (**B**) Basophil activation determined at day 27 by surface expression of CD200R upon stimulation of whole blood with OVA (after subtracting baseline basophil activation). (**C**) Serum OVA-specific IgE levels measured 16 h after oral challenge. (**D**) Plasma cell ($CD138^+B220^-$ of $CD19^-$ cells) frequency assessed in the MLN. (**E**) Serum OVA-specific IgG1 and (**F**) IgA levels measured 16 h after oral challenge. Data are presented as mean ± SEM or as box-and-whisker Tukey plot (in which outliers are shown as separately plotted points) when data were not normally distributed, $n = 6$ in PBS group and $n = 6$-8 in all other groups. For the basophil activation test (**B**), blood samples from two mice were pooled, $n = 3$ in the PBS group and $n = 4$ in all other groups. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups (**A,B,D-F**) or Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups (**C**). OVA, ovalbumin; raw, raw cow's milk; pasteurized, pasteurized cow's milk; skimmed, skimmed raw cow's milk; pasteurized + ALP, pasteurized milk spiked with alkaline phosphatase; MFI, median fluorescence intensity; AU, arbitrary units; MLN, mesenteric lymph nodes; OD, optical density.

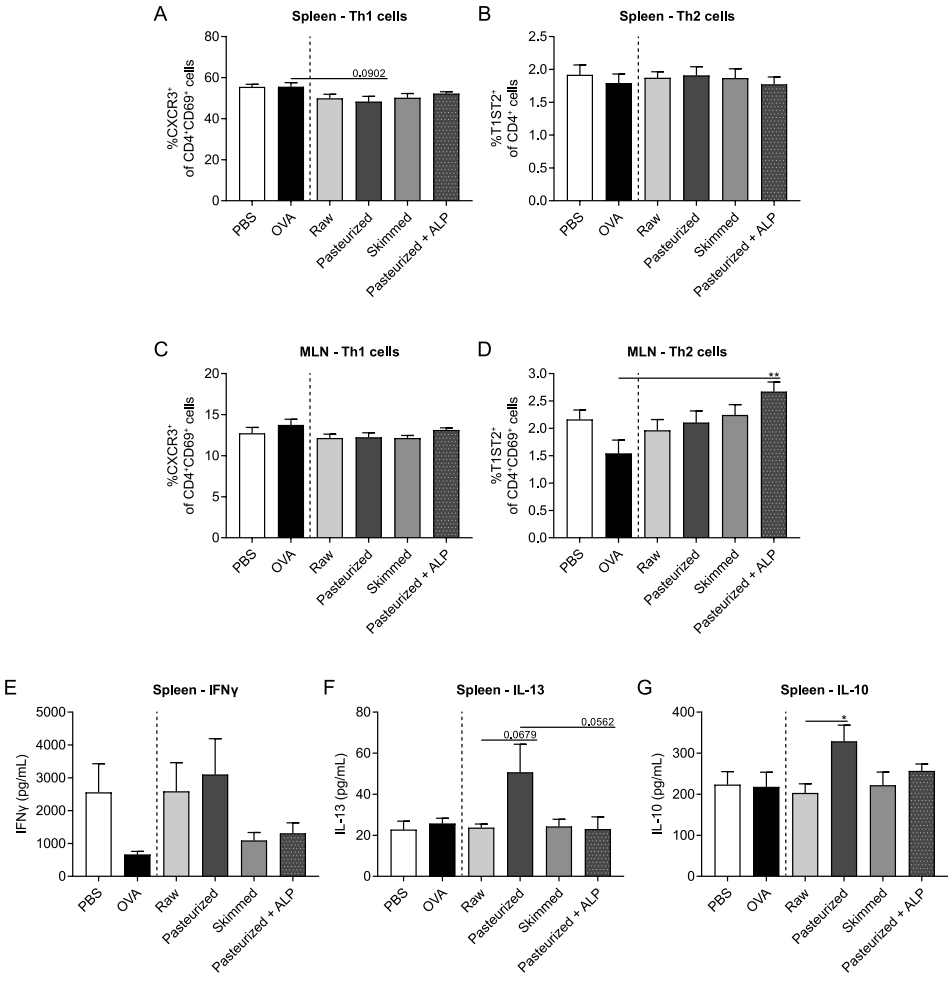


Figure 3. Th2-related cytokine concentrations produced by OVA-stimulated splenocytes were low in raw milk- and skimmed milk-treated mice. (**A**) The percentage of activated Th1 cells (CXCR3$^+$ of CD4$^+$CD69$^+$ cells) and (**B**) Th2 cells (T1ST2$^+$ of CD4$^+$ cells) in the spleen. (**C**) The percentage of activated Th1 cells (CXCR3$^+$ of CD4$^+$CD69$^+$ cells) and (**D**) activated Th2 cells (T1ST2$^+$ of CD4$^+$CD69$^+$) in the MLN. (**E**) IFNγ, (**F**) IL-13, and (**G**) IL-10 concentrations measured in supernatant of *ex vivo* stimulated splenocytes with OVA (stimulated for four days, 37 °C, 5% CO_2). Data are presented as mean ± SEM, n = 6 in PBS group and n = 6-8 in all other groups. *P < 0.05, **P < 0.01, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups. OVA, ovalbumin; raw, raw cow's milk; pasteurized, pasteurized cow's milk; skimmed, skimmed raw cow's milk; pasteurized + ALP, pasteurized milk spiked with alkaline phosphatase; MLN, mesenteric lymph nodes.

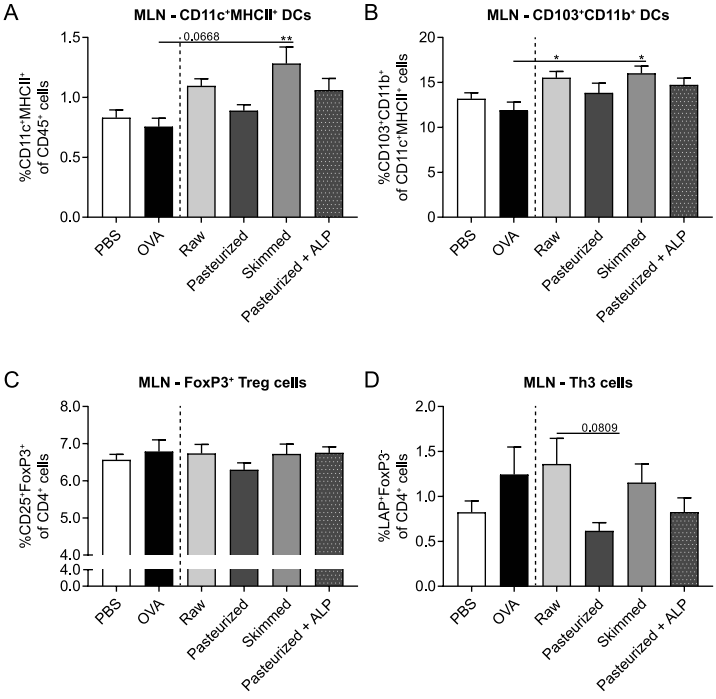


Figure 4. Induction of tolerance-associated cell types in the MLN of raw milk- and skimmed milk-treated mice. (**A**) Percentage of $CD11c^+MHCII^+$ DCs ($CD11c^+MHCII^+$ of $CD45^+$ cells), (**B**) $CD103^+CD11b^+$ DCs ($CD103^+CD11b^+$ of $CD11c^+MHCII^+$ cells), (**C**) $FoxP3^+$ Treg cells ($CD25^+FoxP3^+$ of $CD4^+$ cells), and (**D**) Th3 cells (LAP^+FoxP3^- of $CD4^+$ cells) in the MLN. Data are presented as mean ± SEM, $n = 6$ in PBS group and $n = 5$-8 in all other groups. $^*P < 0.05$, $^{**}P < 0.01$, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups. MLN, mesenteric lymph nodes; OVA, ovalbumin; raw, raw cow's milk; pasteurized, pasteurized cow's milk; skimmed, skimmed raw cow's milk; pasteurized + ALP, pasteurized milk spiked with alkaline phosphatase.

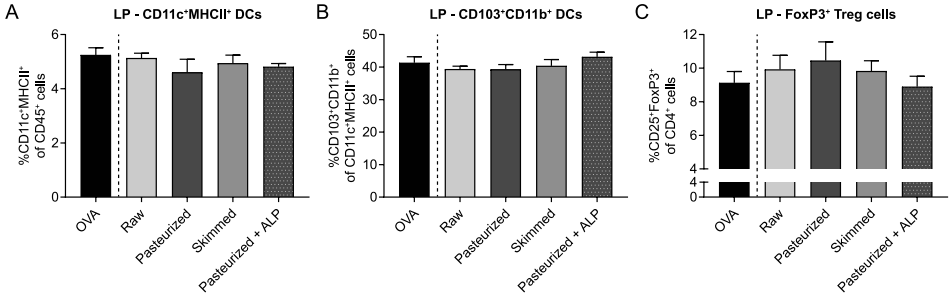


Figure 5. Different milk types did not affect tolerogenic DC and Treg cell frequency in the LP. (**A**) Percentage of CD11c$^+$MHCII$^+$ DCs (CD11c$^+$MHCII$^+$ of CD45$^+$ cells), (**B**) CD103$^+$CD11b$^+$ DCs (CD103$^+$CD11b$^+$ of CD11c$^+$MHCII$^+$ cells), and (**C**) FoxP3$^+$ Treg cells (CD25$^+$FoxP3$^+$ of CD4$^+$ cells) in the LP. Data are presented as mean ± SEM, *n* = 5-6/group. No significant differences were observed. LP, lamina propria; OVA, ovalbumin; raw, raw cow's milk; pasteurized, pasteurized cow's milk; skimmed, skimmed raw cow's milk; pasteurized + ALP, pasteurized milk spiked with alkaline phosphatase.

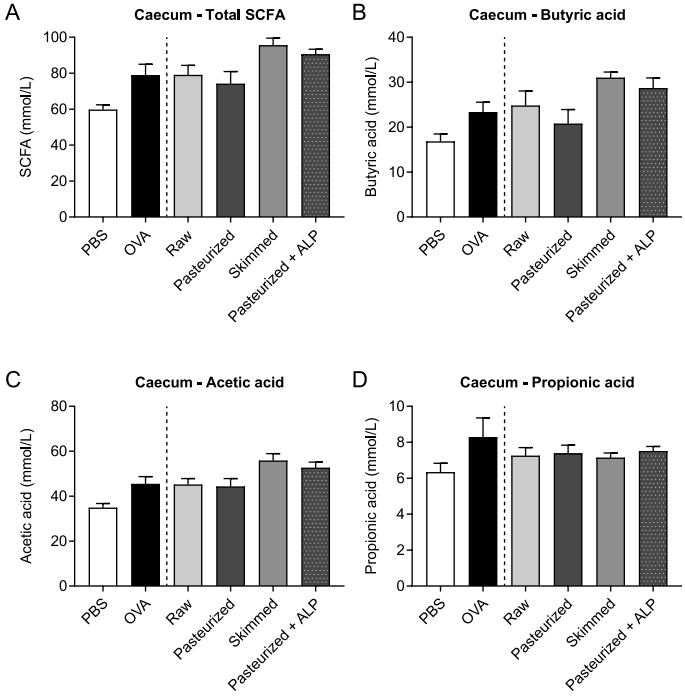


Figure 6. No differences in SCFA concentrations between milk groups. (**A**) Total SCFA concentrations and individual concentrations of (**B**) butyric acid, (**C**) acetic acid, and (**D**) propionic acid measured in caecal content. Data are presented as mean ± SEM, *n* = 6 in PBS group and *n* = 7-8 in all other groups. No significant differences were observed. SCFA, short-chain fatty acids; OVA, ovalbumin; raw, raw cow's milk; pasteurized, pasteurized cow's milk; skimmed, skimmed raw cow's milk; pasteurized + ALP, pasteurized milk spiked with alkaline phosphatase.

DISCUSSION

We previously showed that raw, unprocessed, cow's milk induces tolerance to OVA, an unrelated, non-milk, food allergen, in a murine food allergy model (11). In the present study, we demonstrated that this protective effect is retained after skimming but abolished after pasteurization of the milk. Similar to raw cow's milk, skimmed raw milk reduced the acute allergic skin response after intradermal challenge with OVA. This coincided with low levels of OVA-specific IgE and Th2-related cytokines. An increase in $CD103^{+}CD11b^{+}$ DCs and TGF-β-producing Treg cells in the MLN, both associated with tolerance induction, might underlie the allergy-protective effects of raw and skimmed raw cow's milk. In addition, this study provides a first indication that adding ALP to heat-treated milk might be an interesting preventive strategy since spiking pasteurized milk with ALP restored the allergy-protective effects.

Although several epidemiological studies have shown the potency of raw cow's milk to reduce/prevent allergic diseases (5-9), its consumption is limited due to the potential presence of pathogens. The risk of disease outbreaks by pathogens such as, *Mycobacterium tuberculosis*, *Listeria*, *Salmonella*, *Campylobacter*, Enterohemorrhagic *Escherichia coli*, and Shigatoxigenic *Escherichia coli* is reason for governmental agencies to prohibit the sale of raw cow's milk (12). To prevent this potential risk, milk used for commercial purposes is processed. This means that, upon collection, raw milk undergoes various processing steps like milk fat standardization, homogenization, and heat treatment. These processing steps have profound effects on the milk structure and are shown to be detrimental to the allergy-protective effects (5, 10, 11, 14).

Milk processing predominantly affects the fat content of the milk and the heat-sensitive milk components (21, 22). Since milk processing also abolishes the allergy-protective effects of raw cow's milk, this suggests that these constituents contribute to the observed protection. Indeed, both Wijga *et al.* and Waser *et al*. showed that frequent consumption of products containing milk fat was associated with a reduced asthma risk (8, 23). In addition, Brick *et al.* concluded that part of the asthma-protective effect of raw cow's milk was explained by a higher fat content and particularly, higher *n*-3 polyunsaturated fatty acids levels compared to shop milk (14). However, at the same time, there are also studies where the total fat content was not significantly related to asthma (5). Epidemiological evidence also exists for a potential contribution of heat-sensitive raw milk components. Loss *et al.* demonstrated that raw farm milk, but not boiled farm milk, was inversely associated with asthma, hay fever, and atopy. The heat-sensitive whey protein fraction of raw milk was implied to underlie these effects (5). However, since these are all associations, proof of causality is needed to confirm the protective effects of these different raw milk constituents.

In the present study, we therefore investigated the effect of skimmed raw milk and pasteurized milk in a murine OVA-induced food allergy model. Skimmed milk was as allergy-protective as raw milk, suggesting that the fat content of the milk does not contribute to a large extent to the allergy-protective effects of raw cow's milk. Our results are in contrast with most of the epidemiological findings (8, 14, 23), emphasizing the importance of demonstrating a cause-effect relationship. On the other hand, the discrepancy could also be caused by the fact that these epidemiological studies mainly focused on asthma, whereas our study focused on food allergy. Different disease pathogenesis might underlie the different outcomes.

In contrast to skimming, pasteurization abolished the allergy-protective effects of raw cow's milk in the murine food allergy model used. This is in accordance with epidemiological evidence and with our previous results in a murine asthma model, both showing a loss of protection after heat treatment (5, 10). By comparing milk from the same origin, differing in only one processing step we can conclude with certainty that pasteurization is harmful to the allergy-protective capacity of raw cow's milk. The importance of heat-sensitive milk components, such as proteins, microRNAs, and microbes, is thereby emphasized. Particularly, the heat-sensitive whey protein fraction of raw milk is often mentioned as source of the allergy-protective components. The major whey proteins, α-lactalbumin, β-lactoglobulin, and bovine serum albumin do not have immunomodulatory functionalities that can directly be linked to the allergy-protective effects of raw cow's milk, but several less abundant whey proteins such as immunoglobulins, lactoferrin, TGF-β, and IL-10 theoretically do (24-26).

A first step towards identifying the potential allergy-protective whey proteins was made by Brick *et al.* who investigated the effect of processing intensity on immunologically active milk proteins (27). As expected, a decrease in the number and abundance of detectable native whey proteins was observed with increasing heat load. Interestingly, the subsequent proteomic analysis categorized the milk samples into two clusters; high (boiled, ultra-high temperature processing and extended shelf life) and no/low heat treatment (raw, skimmed, pasteurized) (27). Although pasteurized milk clustered together with raw and skimmed milk, indicating similar native protein patterns, it did not confer protection in our study. One could therefore argue that the overall native protein pattern looks similar but that minor differences still have major consequences for the allergy-protective capacity of the milk. One could also argue that even though pasteurization does not lead to denaturation or chemical modifications of whey proteins, it might lead to loss of functionality.

Although the effect of processing intensity on immunologically active whey proteins is very relevant, it does not provide a direct link to allergic diseases. To provide this link, specific whey proteins can be added to heat-treated milk to see whether they could restore the allergy-

protective effect. As a first proof-of-concept, we spiked pasteurized milk with ALP and we assessed the effects on OVA-induced food allergic symptoms. ALP is probably best known for its function in dairy industry as indicator of successful pasteurization. Upon pasteurization, ALP becomes inactivated and loses its activity, making it an ideal indicator of product safety (28). Since ALP is one of the first bioactive raw milk components losing activity upon heat treatment, it is also a likely allergy-protective candidate. Oral administration of ALP was already shown to be effective in reducing inflammatory diseases (29-32), but whether it can also affect allergic diseases has, to our knowledge, never been studied.

Surprisingly, ALP was able to fully restore the allergy-protective effect in the food allergy model used. On practically every parameter assessed, ALP added to pasteurized milk showed similar protective effects as raw milk and skimmed raw milk. As this was a first proof-of-concept, 10 times higher ALP concentrations than present in raw cow's milk were added to pasteurized milk. We can therefore not yet conclude that ALP is the component underlying the allergy-protective effects of raw cow's milk, but it seems to be a promising candidate to be used as supplement to heat-treated milk.

In addition to the components involved, this study also provides some indication of the underlying mechanisms. The fact that mice orally treated for eight days with raw cow's milk were protected against OVA-induced allergic symptoms indicates that they developed oral tolerance to OVA. This oral tolerance was induced in the absence of the allergen, demonstrating that raw cow's milk has the capacity to induce tolerance via generic immunomodulation. The many immunomodulatory components present in raw cow's milk are likely to create a tolerogenic environment favoring unresponsiveness upon allergen exposure. Raw cow's milk is hypothesized to promote Treg cell development, to modulate the gut microbiome, and to enhance intestinal barrier function (24-26). However, none of these effects have actually been demonstrated after drinking raw milk.

The present study therefore tried to get more insight into some of these proposed mechanisms. Treg cells are identified as key players in inducing and maintaining oral tolerance (33). However, in our study, the percentage of $CD25^{+}FoxP3^{+}$ Treg cells in the MLN was not affected by raw milk treatment. Interestingly, raw milk did increase the Treg subtype secreting TGF-β compared to pasteurized milk. The importance of these Th3 cells is demonstrated in a study showing reduced numbers in the intestine of food allergic children (34).

Induction of $FoxP3^{+}$ Treg cells occurs in the MLN by $CD103^{+}$ DCs under the influence of retinoic acid and TGF-β (35, 36). $CD103^{+}$ DCs originate in the LP and migrate to the MLNs in a CCR7-dependent manner after acquiring antigen (37). This DC trafficking from the intestinal mucosa to the MLNs is crucial for oral tolerance induction (38, 39). Interestingly, while raw milk

exposure did not significantly affect $CD25^+FoxP3^+$ Treg cells in the MLN, it did increase the tolerogenic $CD103^+CD11b^+$ DC subpopulation. Besides promoting the development of $FoxP3^+$ Treg cells, these DCs also induce the expression of gut-homing receptors on the cell surface of $FoxP3^+$ Treg cells (35), indicating that the Treg cells might have migrated to the gut. However, also in the LP, $FoxP3^+$ Treg cell frequency was not increased by raw milk. Examining effects on Treg cell populations directly after raw milk exposure, instead of at the end of the study, might be of importance, since farm milk exposure was previously shown to be associated with increased $FoxP3^+$ Treg cell numbers in children (40).

Regarding the potential immune modulation via the gut microbiome, results were not convincing for raw milk. Caecal SCFA concentrations, as indicator of metabolic activity of the gut microbiome, were not altered compared to other milk groups. However, effects on the gut microbiota itself were not assessed and the timing of measuring SCFA levels might also be crucial in this case. Highest SCFA concentrations, particularly butyric acid and acetic acid, were observed after exposure to skimmed raw milk and ALP. Since oral administration of ALP was previously shown to preserve normal gut microbiome homeostasis (41-43), it is tempting to speculate that this feature contributes to its allergy-protective effect.

A limitation of the current study is the lack of a significant IgE response in OVA-sensitized allergic mice compared to PBS-sensitized control mice. However, although significance was not reached, most of the animals in the OVA group did show higher OVA-IgE levels than animals in the PBS group. We would like to emphasize that serum IgE levels do not always correlate with the severity of the allergic response and that allergic symptoms are not solely induced by IgE (44-46). The acute allergic skin response is the primary parameter of food allergic symptoms in the validated mouse model used. This response is acknowledged as a true acute allergic response and recognized as a translatable readout (47, 48).

In summary, we demonstrated that the suppression of food allergic symptoms by raw cow's milk is retained after skimming but abolished after pasteurization of the milk. The data presented therefore indicate that not the fat content, but the heat-sensitive milk components are underlying the allergy-protective effects of raw cow's milk. The protection by raw and skimmed raw cow's milk was accompanied by an induction of tolerance-associated cell types in the MLN. In addition, we showed that ALP has the capacity to restore the allergy-protective effects abolished by heat treatment. This study thereby provides, for the first time, a direct link between one of the immunologically active whey proteins present in raw cow's milk and the suppression of allergic symptoms. Although its potency and mechanism of action still need to be determined, ALP is a promising raw milk component to be added to heat-treated milk. Hence, this research represents an attractive preventive strategy for allergic diseases as alternative to raw milk consumption.

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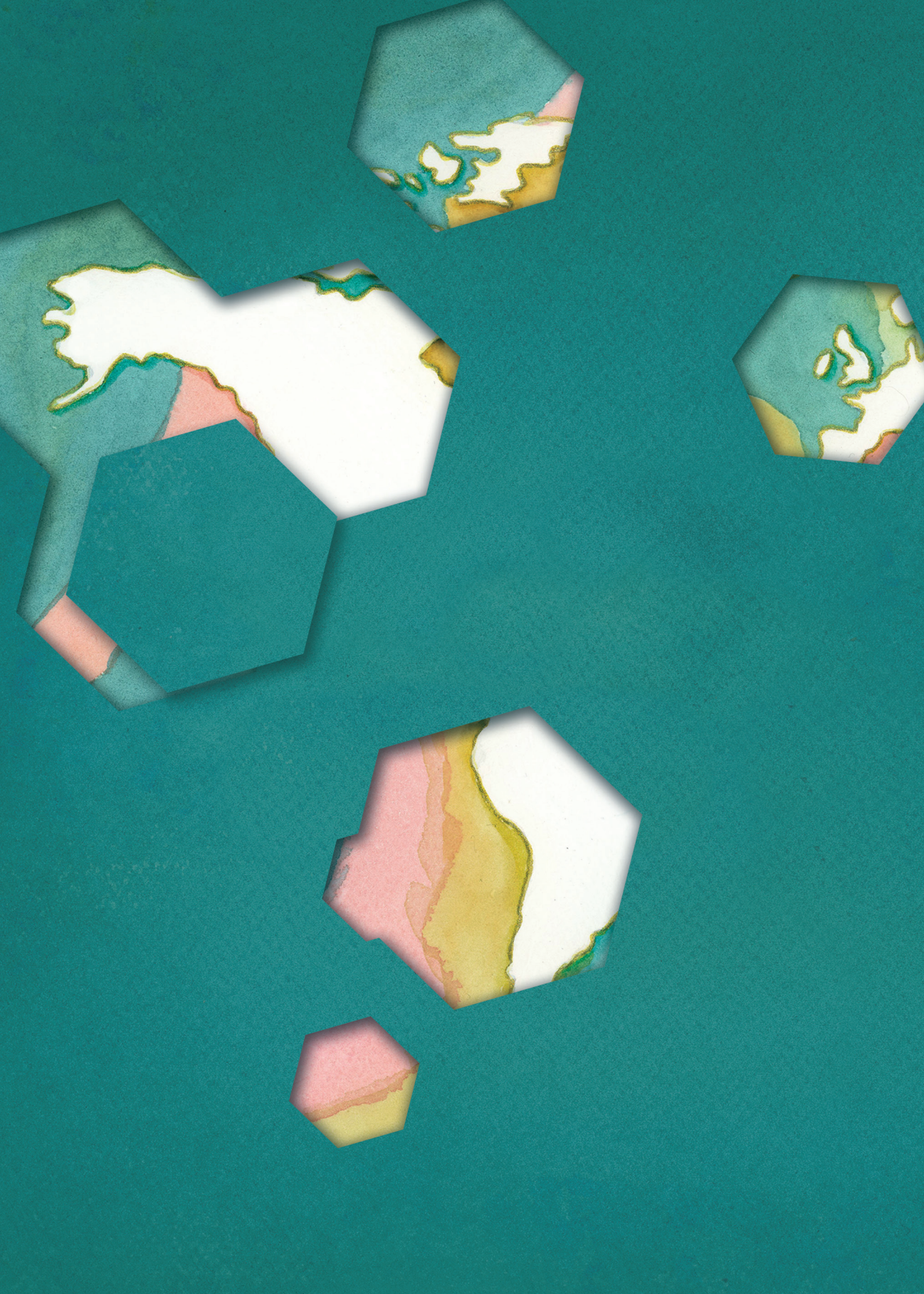
REFERENCES

1. West CE, D'Vaz N, and Prescott SL. Dietary immunomodulatory factors in the development of immune tolerance. *Curr Allergy Asthma Rep* (2011) 11(4): 325-33.
2. Thijs C, Muller A, Rist L, Kummeling I, Snijders BE, Huber M, *et al*. Fatty acids in breast milk and development of atopic eczema and allergic sensitisation in infancy. *Allergy* (2011) 66(1): 58-67.
3. Verhasselt V, Milcent V, Cazareth J, Kanda A, Fleury S, Dombrowicz D, *et al*. Breast milk-mediated transfer of an antigen induces tolerance and protection from allergic asthma. *Nat Med* (2008) 14(2): 170-5.
4. Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, and Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. *Arch Dis Child* (2006) 91(10): 814-9.
5. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
6. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
7. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
8. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, *et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
9. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
10. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T, *et al*. Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model. *Front Immunol* (2017) 8: 1045.
11. Abbring S, Wolf J, Ayechu Muruzabal V, Diks MAP, Alashkar Alhamwe B, Alhamdan F, *et al*. Raw cow's milk reduces allergic symptoms in a murine model for food allergy - A potential role for epigenetic modifications. *Nutrients* (2019) 11(8): 1721.
12. Committee on Infectious Diseases & Committee on Nutrition & American Academy of Pediatrics. Consumption of raw or unpasteurized milk and milk products by pregnant women and children. *Pediatrics* (2014) 133(1): 175-9.
13. Bundesministerium der Justiz und für Verbraucherschutz. Verordnung über die Güteprüfung und Bezahlung der Anlieferungsmilch (Milch-Güteverordnung). (1980) Available from: http://www.gesetze-im-internet.de/milchg_v/index.html Accessed on: 26 March 2019
14. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-1706 e13.
15. Torrero MN, Larson D, Hubner MP, and Mitre E. CD200R surface expression as a marker of murine basophil activation. *Clin Exp Allergy* (2009) 39(3): 361-9.
16. Deurloo DT, van Esch BCAM, Hofstra CL, Nijkamp FP, and van Oosterhout AJ. CTLA4-IgG reverses asthma manifestations in a mild but not in a more "severe" ongoing murine model. *Am J Respir Cell Mol Biol* (2001) 25(6): 751-60.

17. Abbring S, Kusche D, Roos TC, Diks MAP, Hols G, Garssen J, *et al*. 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(7): 1013-25.
18. Bakker-Zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJ, and Bindels JG. 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(5): 783-90.
19. Polukort SH, Rovatti J, Carlson L, Thompson C, Ser-Dolansky J, Kinney SR, *et al*. IL-10 enhances IgE-mediated mast cell responses and is essential for the development of experimental food allergy in IL-10-deficient mice. *J Immunol* (2016) 196(12): 4865-76.
20. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, *et al*. A functionally specialized population of mucosal CD103$^+$ DCs induces Foxp3$^+$ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* (2007) 204(8): 1757-64.
21. Michalski MC and Januel C. Does homogenization affect the human health properties of cow's milk? *Trends Food Sci Tech* (2006) 17(8): 423-37.
22. Niero G, Penasa M, Berard J, Kreuzer M, Cassandro M, and De Marchi M. Technical note: development and validation of an HPLC method for the quantification of tocopherols in different types of commercial cow milk. *J Dairy Sci* (2018) 101(8): 6866-71.
23. Wijga AH, Smit HA, Kerkhof M, de Jongste JC, Gerritsen J, Neijens HJ, *et al*. Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: the PIAMA birth cohort study. *Thorax* (2003) 58(7): 567-72.
24. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
25. Abbring S, Hols G, Garssen J, and van Esch BCAM. Raw cow's milk consumption and allergic diseases - The potential role of bioactive whey proteins. *Eur J Pharmacol* (2019) 843: 55-65.
26. Perdijk O, van Splunter M, Savelkoul HFJ, Brugman S, and van Neerven RJJ. Cow's milk and immune function in the respiratory tract: potential mechanisms. *Front Immunol* (2018) 9: 143.
27. Brick T, Ege M, Boeren S, Bock A, von Mutius E, Vervoort J, *et al*. Effect of processing intensity on immunologically active bovine milk serum proteins. *Nutrients* (2017) 9(9): 963.
28. Rankin SA, Christiansen A, Lee W, Banavara DS, and Lopez-Hernandez A. Invited review: the application of alkaline phosphatase assays for the validation of milk product pasteurization. *J Dairy Sci* (2010) 93(12): 5538-51.
29. Beumer C, Wulferink M, Raaben W, Fiechter D, Brands R, and Seinen W. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. *J Pharmacol Exp Ther* (2003) 307(2): 737-44.
30. Tuin A, Poelstra K, de Jager-Krikken A, Bok L, Raaben W, Velders MP, *et al*. Role of alkaline phosphatase in colitis in man and rats. *Gut* (2009) 58(3): 379-87.
31. Whitehouse JS, Riggle KM, Purpi DP, Mayer AN, Pritchard KA, Jr., Oldham KT, *et al*. The protective role of intestinal alkaline phosphatase in necrotizing enterocolitis. *J Surg Res* (2010) 163(1): 79-85.
32. Lalles JP. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. *Nutr Rev* (2010) 68(6): 323-32.
33. Akdis M, Blaser K, and Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* (2005) 116(5): 961-8; quiz 969.

34. Perez-Machado MA, Ashwood P, Thomson MA, Latcham F, Sim R, Walker-Smith JA, *et al*. Reduced transforming growth factor-beta1-producing T cells in the duodenal mucosa of children with food allergy. *Eur J Immunol* (2003) 33(8): 2307-15.
35. Scott CL, Aumeunier AM, and Mowat AM. Intestinal CD103$^+$ dendritic cells: master regulators of tolerance? *Trends Immunol* (2011) 32(9): 412-9.
36. Mucida D, Pino-Lagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, *et al*. Retinoic acid can directly promote TGF-beta-mediated Foxp3$^+$ Treg cell conversion of naive T cells. *Immunity* (2009) 30(4): 471-2; author reply 472-3.
37. Jang MH, Sougawa N, Tanaka T, Hirata T, Hiroi T, Tohya K, *et al*. CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. *J Immunol* (2006) 176(2): 803-10.
38. Worbs T and Forster R. A key role for CCR7 in establishing central and peripheral tolerance. *Trends Immunol* (2007) 28(6): 274-80.
39. Spahn TW, Weiner HL, Rennert PD, Lugering N, Fontana A, Domschke W, *et al*. Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. *Eur J Immunol* (2002) 32(4): 1109-13.
40. Lluis A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D, *et al*. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol* (2014) 133(2): 551-9.
41. Bates JM, Akerlund J, Mittge E, and Guillemin K. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe* (2007) 2(6): 371-82.
42. Malo MS, Alam SN, Mostafa G, Zeller SJ, Johnson PV, Mohammad N, *et al*. Intestinal alkaline phosphatase preserves the normal homeostasis of gut microbiota. *Gut* (2010) 59(11): 1476-84.
43. Lalles JP. Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr Rev* (2014) 72(2): 82-94.
44. Schouten B, van Esch BCAM, van Thuijl AO, Blokhuis BR, Groot Kormelink T, Hofman GA, *et al*. Contribution of IgE and immunoglobulin free light chain in the allergic reaction to cow's milk proteins. *J Allergy Clin Immunol* (2010) 125(6): 1308-14.
45. Sampson HA, Aceves S, Bock SA, James J, Jones S, Lang D, *et al*. Food allergy: a practice parameter update-2014. *J Allergy Clin Immunol* (2014) 134(5): 1016-25 e43.
46. Schouten B, van Esch BCAM, Hofman GA, van den Elsen LW, Willemsen LE, and Garssen J. Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. *Int Arch Allergy Immunol* (2008) 147(2): 125-34.
47. van Esch BCAM, Schouten B, Hofman GA, van Baalen T, Nijkamp FP, Knippels LM, *et al*. Acute allergic skin response as a new tool to evaluate the allergenicity of whey hydrolysates in a mouse model of orally induced cow's milk allergy. *Pediatr Allergy Immunol* (2010) 21(4 Pt 2): e780-6.
48. van Esch BCAM, van Bilsen JH, Jeurink PV, Garssen J, Penninks AH, Smit JJ, *et al*. 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(1): 95-102.



CHAPTER 6

Loss of allergy-protective capacity of raw cow's milk after heat treatment coincides with loss of immunologically active whey proteins – Native proteomics combined with a functional readout for allergic diseases

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ABSTRACT

The allergy-protective capacity of raw cow's milk was demonstrated to be abolished after heat treatment. The heat-sensitive whey protein fraction of raw milk is often implied to be the source of this allergy-protective effect, but a direct link between these proteins and the protection against allergic diseases is missing. This study therefore aimed at investigating the mechanistic relation between heat damage to whey proteins and allergy development. Raw cow's milk was heated for 30 min at 50, 60, 65, 70, 75, or 80 °C and the native whey protein profile of these differentially heated milk samples was determined using LC-MS/MS-based proteomics. Changes in the native protein profile were subsequently related to the capacity of the milk to prevent the development of ovalbumin-induced food allergy in a murine animal model. A substantial loss of native whey proteins, as well as extensive protein aggregation, was observed from 75 °C. However, whey proteins with immune-related functionalities already started to denature from 65 °C, which coincided with the temperature at which a loss of allergy protection was observed in the murine model. Of the immunologically active whey proteins, complement C7, monocyte differentiation antigen CD14, and polymeric immunoglobulin receptor concentrations decreased significantly at this temperature, although several other of these proteins also showed a decrease around 65 °C. The current study demonstrates that immunologically active whey proteins that denature around 65 °C are of importance for the allergy-protective capacity of raw cow's milk and thereby provides key knowledge for the development of microbiologically safe alternatives to raw cow's milk.

INTRODUCTION

Allergic diseases are a serious health problem. They affect the lives of more than a billion people worldwide and their prevalence is expected to rise to four billion by 2050. Because of the large number of people affected, society faces huge health care costs and major socio-economic consequences (1).

The vast increase in the prevalence of allergic diseases is mainly evident in industrialized countries and is often linked to the Western lifestyle (2, 3). This lifestyle is accompanied by the loss of rural living conditions resulting in a decreased microbial exposure in early life. In accordance with this so-called 'hygiene hypothesis', epidemiological studies have consistently shown that children growing up on a farm are at a significantly lower risk of developing allergic diseases than children living in the same rural area but not growing up on a farm (3-9). Several farm exposures have been associated with this protective 'farm effect' (10-13), but particularly the consumption of raw, unprocessed, cow's milk is of interest, since its effect was found to be independent of living on a farm, suggesting that a general, non-farming, population might equally benefit from the consumption of raw cow's milk (13-16). Raw cow's milk consumption can thus be considered a possible allergy-preventive measure. However, due to the potential presence of pathogens, its consumption is discouraged by regulatory agencies (17).

A wide range of components have been hypothesized to contribute to the allergy-protective effects of raw cow's milk (18-20). Since recent research clearly demonstrates a loss of protection upon heat treatment, heat-sensitive raw milk components, like proteins, are the most likely candidates (15, 16, 21, 22). Among the milk proteins, mainly whey proteins are susceptible to heat treatment (23). Heating of these proteins induces processes like denaturation and aggregation which results in structural alterations that can lead to loss of functionality (24-26). Particularly immunologically active whey proteins, like lactoferrin and immunoglobulins, are heat-sensitive (27). Since these components have immune-related functionalities that can be linked to the allergy-protective effects of raw cow's milk, abolishing them by heat treatment could be detrimental (19). In addition to destroying immunologically active whey proteins, heat treatment can also induce the formation of new protein structures. Heating, for example, leads to aggregation of β-lactoglobulin and α-lactalbumin, both with themselves as well as with casein micelles (25, 26). Aggregation of these whey proteins has been demonstrated to result in a shift in uptake from enterocytes to Peyer's patches thereby leading to increased immunogenicity (28). A better understanding of the heat sensitivity of whey proteins is key for the development of new processing steps, that may lead to a safe and protective product in the future.

Although the effect of milk processing on whey proteins is widely studied, a direct link demonstrating the consequences on the allergy-protective effects associated with raw milk is lacking. Providing this link is crucial to gain more insight in the actual whey protein(s) involved in the allergy-protective effects of raw cow's milk. In the present study, native proteomics was therefore combined with a functional readout for allergic diseases. Since the many proteins present in the whey fraction of raw milk all have a different heat sensitivity, small differences in heating temperature (50, 60, 65, 70, 75, and 80 °C, chosen to be ranging from almost no to almost full whey protein denaturation) were used to gain more insight in the denaturation profile of specific whey proteins. Changes in the native protein profile of these differentially heated milk samples were subsequently related to the capacity of the milk to prevent the development of ovalbumin (OVA)-induced food allergy in a murine animal model. This study hereby aimed at achieving a better understanding of the underlying mechanism between heat damage to whey proteins and allergy development.

MATERIALS & METHODS

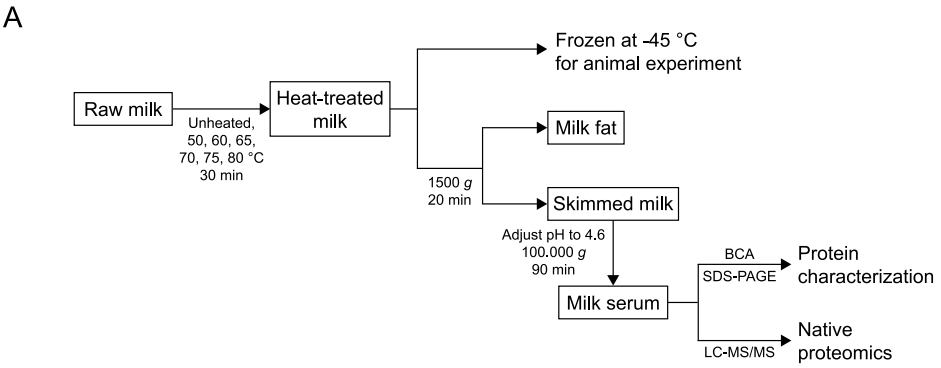
Mice

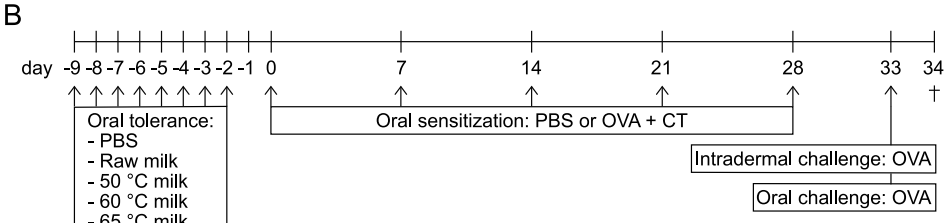
Specific pathogen-free, three-week-old, female C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, mice were randomly assigned to the control or experimental groups. They were housed at the animal facility of Utrecht University (Utrecht, The Netherlands) in filter-topped makrolon cages (one cage/group, n = 6-8/cage) on a 12 h light/dark cycle with access to food ('Rat and Mouse Breeder and Grower Expanded'; Special Diet Services, Witham, UK) and water *ad libitum*. Animal procedures were approved by the Ethical Committee for Animal Research of the Utrecht University and conducted in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes (AVD108002015346).

Milk collection and heat treatment

Raw cow's milk was collected from a biodynamic dairy farm legally allowed to sell raw milk (organic 'Vorzugsmilch' (29); Hof Dannwisch, Horst, Germany). Upon collection, part of the raw milk was heated to obtain the 50 °C, 60 °C, 65 °C, 70 °C, 75 °C, and 80 °C milk. For each of these heat treatments, raw milk was divided into three 50 mL aliquots. Two of these aliquots were used for the animal experiment and the milk protein analysis, whereas the other aliquot was used to monitor the temperature during heat treatment. Aliquots were placed in a water bath when the water reached the target temperature. The temperature of the milk was subsequently monitored and as soon as it reached the target temperature (which on average took around 15 min), heating was continued for 30 min.

After these 30 min, the milk was cooled on ice. The milk used for the animal experiment was then aliquoted and stored at -20 °C until further use. The milk used for protein analysis was immediately used to obtain milk serum. An overview of all milk processing steps is shown in **Figure 1A**.





Group	*n*	Tolerance	Sensitization	Challenge
PBS	6	PBS	PBS + CT	OVA
OVA	8	PBS	OVA + CT	OVA
Raw milk	8	Raw milk	OVA + CT	OVA
50 °C milk	8	50 °C milk	OVA + CT	OVA
60 °C milk	8	60 °C milk	OVA + CT	OVA
65 °C milk	8	65 °C milk	OVA + CT	OVA
70 °C milk	8	70 °C milk	OVA + CT	OVA
75 °C milk	8	75 °C milk	OVA + CT	OVA
80 °C milk	8	80 °C milk	OVA + CT	OVA

Figure 1. Schematic overview of the experimental setup (**A**) Flow chart of the various milk processing steps. (**B**) Experimental timeline of the animal study. OVA, ovalbumin; CT, cholera toxin.

Milk serum preparation

After heat treatment, milk samples used for protein analysis were skimmed by centrifugation at 1500 *g* for 20 min at 10 °C (Avanti J-26 XP, rotor 25.15; Beckman Coulter, Miami, FL, USA). Caseins and denatured proteins were subsequently removed as described previously (30). Briefly, milk samples were acidified by drop-wise addition of 1 M HCl under stirring, until a pH of 4.6 was reached. The samples were then kept at 4 °C for 30 min to equilibrate. The acidified skim milk was ultracentrifuged at 100,000 *g* for 90 min at 30 °C (Beckman Optima L-60, rotor

70 Ti; Beckman Coulter). After ultracentrifugation, samples were separated into three phases. The top layer was remaining milk fat, the middle layer was milk serum with native milk proteins, and the bottom layer (pellet) was micellar casein with denatured/aggregated proteins. Milk serum was collected and used for further analysis as described below.

Protein characterization by BCA assay and SDS-PAGE

The soluble protein content remaining in the native milk serum was determined by using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Paisley, Scotland). In addition, milk serum samples were loaded onto an SDS-PAGE and proteins were visualized using Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad, Veenendaal, The Netherlands) to determine the remaining presence of the most abundant milk proteins in the native milk serum. For the reducing SDS-PAGE, milk serum was diluted 5× with distilled water (for raw milk serum 4 µg protein was loaded; for heated milk serum the amount of protein loaded depended on the amount of native proteins remaining in the milk serum). Two microliters of each milk serum sample were then heated at 70 °C for 10 min with 5 µL NuPAGE® LDS Sample Buffer (Thermo Fisher Scientific), 2 µL NuPAGE® Sample Reducing Agent (Thermo Fisher Scientific) and 13 µL water. Subsequently, samples (10 µL) and a PageRuler Prestained Protein Ladder (Thermo Fisher Scientific), ranging from 10 kDa to 180 kDa, were loaded onto a NuPAGE® 10% Bis-Tris Protein Gel (Thermo Fisher Scientific). Electrophoresis was performed at room temperature for approximately 120 min at 120 V (XCell SureLock Mini-Cell; Thermo Fisher Scientific) in NuPAGE® MES SDS Running Buffer (Thermo Fisher Scientific). After electrophoresis, the gel was stained for 1 h with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad) and then destained for 4 h with washing buffer (10% (v/v) ethanol and 7.5% (v/v) acetic acid) while mildly shaking at room temperature. For the non-reducing SDS-PAGE, skim milk samples and milk serum samples were prepared without dithiothreitol (DTT), and running buffer was prepared without antioxidant. The rest of the procedure was performed as described above for the reducing SDS-PAGE. After destaining, the gels were scanned using the ChemiDoc XRS+ Imaging System (Bio-Rad) with Image Lab Software (Bio-Rad).

Sample preparation for proteomics

The Filter Aided Sample Preparation (FASP) method was carried out according to Wiśniewski *et al.* (31), with adaptations according to Hettinga *et al.* and Lu *et al.* (32, 33). In brief, milk serum samples were diluted in 100 mM Tris/HCl, pH 8.0, in 0.5 mL low-binding microcentrifuge tubes (Eppendorf, Hamburg, Germany), to get a protein concentration of approximately 1 µg/µL. After adding 10 µL 150 mM DTT, samples were incubated for 20 min at 45 °C. They were then cooled to room temperature and subsequently centrifuged at 13,524 *g* for 1 min. Reduced samples (10 µg) were added to the middle of 169 µL 100 mM Tris/HCl, pH 8.0, containing 8 M urea in low-binding microcentrifuge tubes. After adding 20 µL 200 mM AcrylAmide (Sigma-Aldrich,

Zwijndrecht, The Netherlands), samples were incubated for 10 min at room temperature while mildly shaking. Part of each sample (100 µL) was then transferred to a Pall 3K Omega filter (10-20 kDa cut-off; Pall Corporation, Port Washington, NY, USA) and centrifuged at 13,524 *g* for 30 min. Afterwards, 110 µL 0.05 M NH_4HCO_3 (ABC) was added to the filter unit followed by centrifugation at 13,524 *g* for 30 min. The filter unit was then transferred to a new low-binding microcentrifuge tube and 100 µL ABC containing 0.5 µg trypsin (Roche, Penzburg, Germany) was added. The filters were incubated for 2 h at 45 °C and subsequently centrifuged at 13,524 *g* for 30 min. Finally, the filter was removed and 6 µL trifluoroacetic acid (TFA; Sigma-Aldrich) was added to adjust the pH of the samples to around 2. These samples were then ready for LC-MS/MS.

Proteomics by LC-MS/MS

The LC-MS/MS parameters used were the same as described previously (34, 35). Samples were analyzed by injecting 18 µL of trypsin-digested milk serum protein fractions in a 0.10 x 30 mm Magic C18AQ 200A 5 µm beads (Michrom BioResources Inc., Auburn, CA, USA) pre-concentration column (prepared in house) at a maximum pressure of 800 bar. Peptides were eluted from the pre-concentration column onto a 0.10 x 200 mm ReproSil-Pur 120 C18-AQ 1.9 µm beads analytical column with an acetonitrile gradient at a flow of 0.5 µL/min, using gradient elution from 8% to 33% acetonitrile in water with 0.5 v/v% acetic acid in 50 min. The column was washed using an increase in the percentage acetonitrile to 80% (with 20% water and 0.5 v/v% acetic acid in the acetonitrile and water) in 3 min. Between the pre-concentration and analytical column, an electrospray potential of 3.5 kV was applied directly to the eluent through a stainless steel needle fitted into the waste line of the micro cross. Full scan positive mode FTMS spectra were measured between m/z 380 and 1400 on an LTQ-Orbitrap XL (Thermo Electron, San Jose, CA, USA). CID-fragmented MS/MS scans of the four most abundant doubly- and triply-charged peaks in the FTMS scan were recorded in data-dependent mode in the linear trap (MS/MS threshold = 5.000).

Data analysis proteomics

Each run with all MS/MS spectra obtained was analyzed with MaxQuant 1.6.0.1 with the Andromeda search engine (36). Trypsin was set as the digestion enzyme. Carbamidomethylation of cysteines was set as a fixed modification. Oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modifications for both identification and quantification. The bovine (taxonomy ID: 9913) reference database for peptide and protein searches was downloaded as fasta file from Uniprot with reverse sequences generated by MaxQuant (fasta file downloaded from Uniprot in 2013 (37)). A set of 31 protein sequences of common contaminants was used as well, which included Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). A

maximum of two missed cleavages were allowed and a mass deviation of 0.5 Da for fragment MS/MS peaks, and 20 ppm and 4.5 ppm for the peptide MS peaks during the first and main search, respectively. The false discovery rate (FDR) was set to 1% on both peptide and protein level. The length of peptides was set to at least seven amino acids. Finally, proteins were identified based on minimally two distinct peptides of which at least one unique and at least one unmodified. The intensity based absolute quantification (iBAQ) algorithm calculates the sum of all peptide intensities dived by the number of theoretically observable tryptic peptides, which has been reported to have a good correlation with known absolute protein concentrations over at least four orders of magnitude (38). The iBAQ values can thus be used as an indication for the absolute amount of protein in the samples and was therefore used to show the quantitative effect of heating on milk proteins. The proteomics data were further analyzed by Perseus 1.6.2.3 (39).

Experimental design animal study

A schematic representation of the experimental timeline is depicted in **Figure 1B**. Prior to the start of the study, mice were habituated to the laboratory conditions for six days. After this acclimatization period, mice were orally exposed, by using a blunt needle, to 0.5 mL raw milk, 50 °C milk, 60 °C milk, 65 °C milk, 70 °C milk, 75 °C milk, 80 °C milk, or PBS for eight consecutive days (days -9 to -2). Subsequently, mice (n = 8/group) were orally (by means of gavage) sensitized to 20 mg of the hen's egg protein OVA (20 mg/0.5 mL PBS; grade V; Sigma-Aldrich) using 15 µg cholera toxin (CT; List Biological Laboratories, Campbell, CA, USA) as an adjuvant (days 0, 7, 14, 21, and 28). Sham-sensitized control mice (n = 6) received CT alone (15 µg/0.5 mL PBS). OVA solutions were passed through Pierce High Capacity Endotoxin Removal Resin (Thermo Fisher Scientific) to remove lipopolysaccharide. Five days after the last sensitization (day 33), all mice were both intradermally and orally challenged with OVA (10 µg/20 µL PBS and 50 mg/0.5 mL PBS respectively). Sixteen hours after the oral challenge (day 34), a blood sample was taken and mice were killed by cervical dislocation.

Assessment of the acute allergic skin response

All mice were intradermally challenged in both ear pinnae with OVA (10 µg OVA/20 µL PBS) to determine the magnitude of the acute allergic skin response. The acute allergic skin response is expressed as Δ ear swelling (µm) and was calculated by subtracting mean basal ear thickness from mean ear thickness 1 h after intradermal challenge. Ear thickness at both timepoints was measured in duplicate for each ear using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). Measurements were carried out by a researcher blinded to treatment. To be able to perform the intradermal challenge and both ear measurements, mice were anesthetized using inhalation of isoflurane (Abbott, Breda, The Netherlands).

Detection of OVA-specific IgE in serum

Blood collected via cheek puncture prior to sacrifice (day 34) was centrifuged at 10,000 rpm for 10 min to obtain serum. Serum samples were stored at -20 °C until analysis of OVA-specific IgE levels by means of ELISA as described elsewhere (22). Results are expressed in arbitrary units, calculated based on an internal standard curve of pooled sera.

Flow cytometric analysis of immune cells

Lymphocytes were isolated from spleen by crushing tissue through a 70 µm nylon cell strainer and removing red blood cells using lysis buffer (8.3 g NH_4Cl, 1 g KHC_3O, and 37.2 mg EDTA dissolved in 1 L demi water, filter sterilized). The obtained single cell suspensions were resuspended in PBS/1% bovine serum albumin (BSA; Sigma-Aldrich). For flow cytometric analysis, cells (0.5-1 × 10^6 cells/well) were subsequently incubated for 15 min on ice with anti-mouse CD16/CD32 (Mouse BD Fc Block; BD Biosciences, Alphen aan de Rijn, The Netherlands) in PBS/1% BSA/5% fetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands) buffer to block non-specific binding sites. Cells were then extracellularly stained with the following antibodies (all purchased from eBioscience, Breda, The Netherlands, unless otherwise stated): CD4-PerCP-Cy5.5, CXCR3-PE, T1ST2-FITC (MD Bioproducts, St. Paul, MN, USA), CD25-Alexa Fluor® 488, latency-associated peptide (LAP)-PE-Cy7, F4/80-APC-eFluor® 780, CD11c-PerCP-Cy5.5, MHCII-FITC, or CD45-PE-Cy7 for 30 min on ice. Fixable Viability Dye-eFluor® 780 (eBioscience) was used according to manufacturer's instructions to exclude dead cells from analysis. Cells that only needed to be stained extracellularly were fixed using IC Fixation Buffer (eBioscience). Cells also to be stained intracellularly were fixed and permeabilized using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's protocol and then stained with FoxP3-APC or IL-10-PE (eBioscience). Fluorescently stained cells were measured with the FACS Canto II (BD Biosciences) and analyzed using FlowLogic Software (Inivai Technologies, Mentone, Australia). Isotype controls were used for each antibody and cut-off gates for positivity were determined with fluorescence-minus-one controls. To detect LAP expression and IL-10 production, spleen-derived lymphocytes were polyclonally stimulated with anti-CD3 (10 µg/mL)/CD28 (1 µg/mL; eBioscience) for 24 h at 37 °C and 5% CO_2, of which the last 4 h in the presence of monensin (eBioscience), prior to staining.

Statistical analysis

Statistical analysis related to the protein analysis was performed using R 3.5.0 (40). Packages 'ggplot2', 'gplots', and 'ggbiplot' were used for graphical representation of the data. Results on the total native whey protein content of the different heat-treated milk samples are presented as mean ± SD of triplicates. Dunnett's multiple comparisons test was used to identify statistical differences between raw and heated milk samples. The hierarchical clustered heat maps, based on Z score normalized iBAQ values of the protein profiles, were created using the R package 'gplots'. Hierarchical clustering of both milk samples

and proteins was performed using the complete agglomeration method and a Euclidean distance metric. Principal component analysis (PCA) was performed based on iBAQ values of the identified proteins using the R package 'prcomp' and plotted using the package 'ggbiplot'. For the statistical analyses related to the animal experiment, GraphPad Prism software (version 7.03; GraphPad Software, San Diego, CA, USA) was used. These data are presented as mean ± SEM or as box-and-whisker Tukey plots when data were not normally distributed. Differences between pre-selected groups were statistically determined using one-way ANOVA, followed by Bonferroni's multiple comparisons test. For OVA-specific IgE levels, the Kruskal-Wallis test followed by Dunn's multiple comparisons test for pre-selected groups was used since data were not normally distributed. For all results, $P < 0.05$ was considered statistically significant.

RESULTS

Substantial loss of native whey proteins at heating temperatures of 70 °C and above

To determine the remaining amount of native whey proteins present in milk after various heat treatments, the protein concentration of the native milk serum was determined. The results of the BCA assay show that the total native protein content gradually decreased from raw, unheated, milk to milk heated at 80 °C (**Figure 2A**). However, a significant decrease in native whey protein concentration was only observed from 70 °C. To get a first indication about the effect of the different heat treatments on the most abundant whey proteins, milk serum was analyzed with a reducing SDS-PAGE and proteins were visualized using Coomassie Brilliant Blue. The two major whey proteins present in cow's milk, β-lactoglobulin and α-lactalbumin, denatured at 80 °C, as demonstrated by reduced protein band intensities on the reducing SDS-PAGE (**Figure 2B**). For BSA, denaturation occurred around 75 °C (**Figure 2B**). For whey proteins with immunomodulatory functionalities, such as immunoglobulins and lactoferrin, denaturation already happened at 70 and 65 °C, respectively (**Figure 2B**). In addition, heat-induced aggregate formation through disulfide interchange, in both skim milk and milk serum samples was assessed using a non-reducing SDS-PAGE. As shown by the non-reducing SDS-PAGE of milk samples, proteins started to aggregate from 75 °C and aggregates were more abundantly present in milk heated at 80 °C (**Figure 2C**). As expected, aggregates were not visible on the non-reducing SDS-PAGE of milk serum samples, since they were removed during acidified ultracentrifugation (**Figure 2D**).

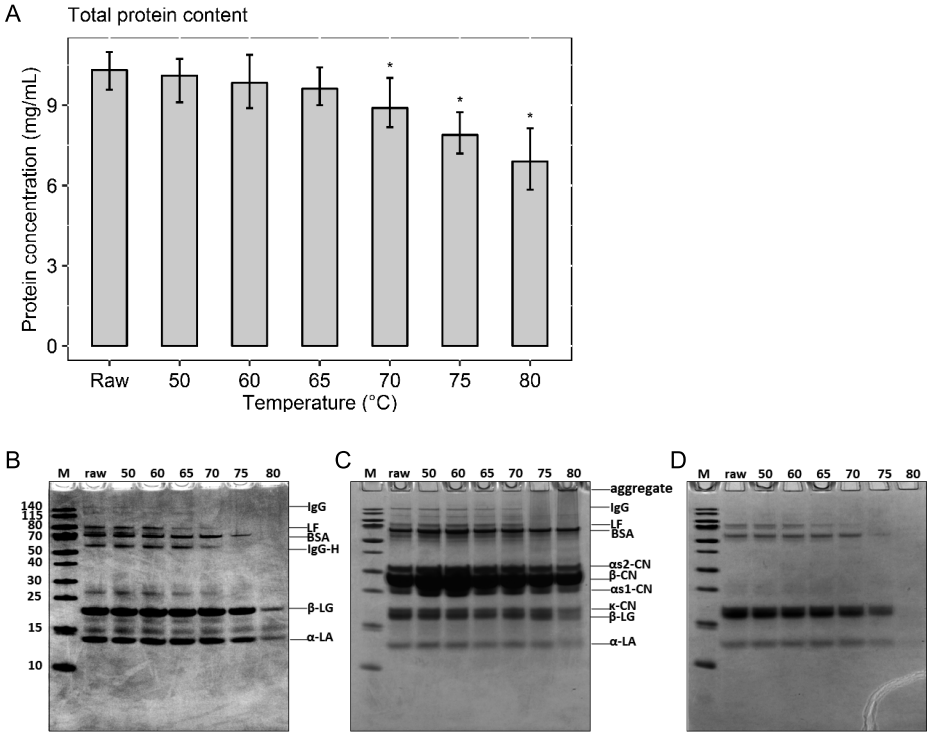


Figure 2. Reduced native whey protein concentration after heating at 70 °C or higher. (**A**) Total protein concentration in native milk serum after different heat treatments. (**B**) Reducing SDS-PAGE of the different native milk serum samples after heat treatment and (**C**) non-reducing SDS-PAGE of the different skim milk samples and (**D**) milk serum samples, with the main proteins that can be identified on the gel indicated. Protein concentrations are presented as mean ± SD of triplicates. *$P < 0.05$ compared to raw milk as analyzed with Dunnett's multiple comparisons test. M, marker; raw, raw cow's milk; 50-80 °C, raw cow's milk heated at the indicated temperature for 30 min; IgG, immunoglobulin G; LF, lactoferrin; BSA, bovine serum albumin; IgG-H, heavy chain of immunoglobulin G; αs2-CN, αs2-casein; β-CN, β-casein; αs1-CN, αs1-casein; κ-CN, κ-casein; β-LG, β-lactoglobulin; α-LA, α-lactalbumin.

Cluster analysis of native protein profile separated 75 and 80 °C from lower heating temperatures

To further analyze the impact of heating on the native whey protein composition, proteomics was performed by means of LC-MS/MS. As expected, results showed that the number of denatured proteins increased with increasing heating intensity, from 1 protein compared to raw milk at 50 °C, to 112 proteins at 80 °C. A sharp increase in the amount of denatured proteins from 73 to 112 was observed between 75 and 80 °C. A heat map was subsequently created which visualized the native protein profile of the different heat-treated milk samples. According to the clusters presented in the heat map, the native protein profile of samples

heated at 75 and 80 °C differed from samples heated at lower temperatures (**Figure 3**). No clear distinction could be made among samples heated below 75 °C (**Figure 3**). Therefore, a PCA plot was created to determine whether the differentially heat-treated milk groups could be distinguished (**Figure 4**). Based on principal component 1, which accounts for 57.5% of the total variation, milk samples heated at 75 and 80 °C differed from samples heated at lower temperatures (**Figure 4**). Unheated and < 70 °C heat-treated milk groups differed from each other mainly by principal component 2, which accounted for 9.9% of the total variation (**Figure 4**). Milk heated at 50 and 60 °C could not be distinguished from each other (**Figure 4**).

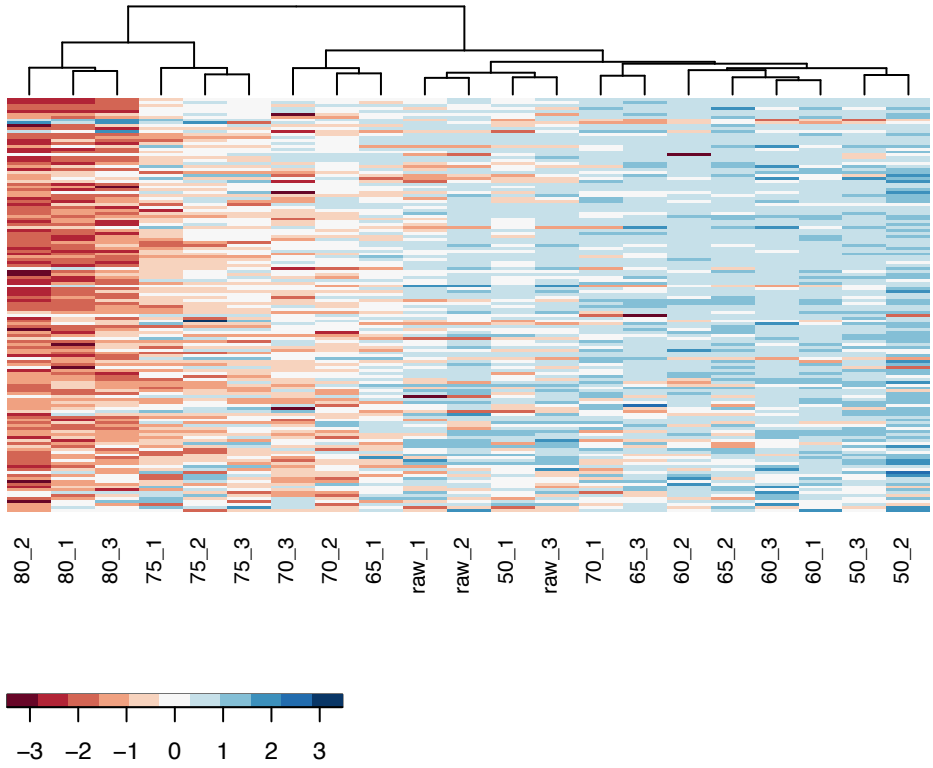


Figure 3. Hierarchical cluster analysis separated 75 and 80 °C from lower heating temperatures based on native proteomics pattern. Protein profile of heated milk serum samples based on Z score normalized iBAQ values. Columns reflect individual samples. The number indicates the heating temperature, while the number after the underscore indicates the replicate. Rows reflect individual proteins. Hierarchical clustering was performed using complete agglomeration method and a Euclidean distance metric. The color scale reflects the iBAQ value-based Z score.

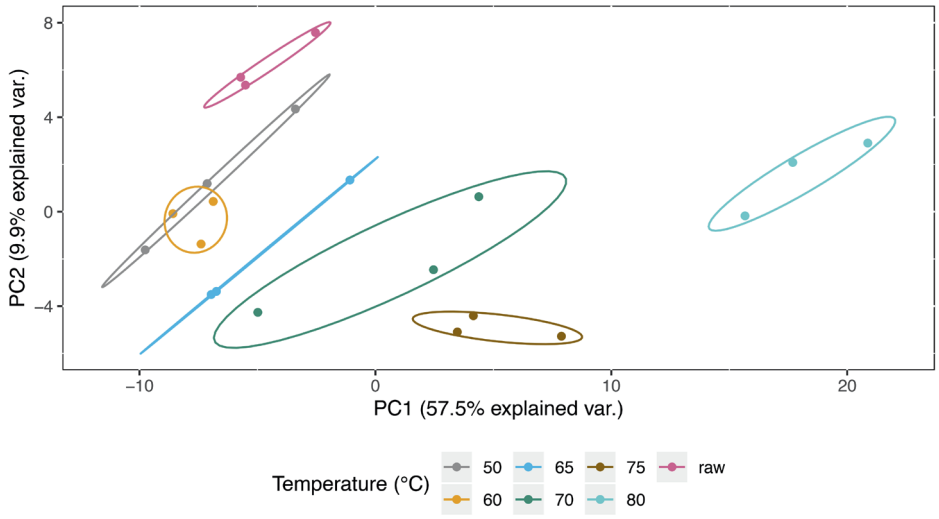


Figure 4. Principal Component Analysis (PCA) distinguished different heat-treated milk groups based on native protein profile. Milk heated at 50 and 60 °C could not be distinguished from each other. Colored symbols refer to the seven milk groups tested. The first two principal axes explained 67.4% of the variance. The variation (%) explained by each PCA axis is in parentheses. PC1, principal component 1; PC2, principal component 2.

Loss of allergy protection in murine OVA-induced food allergy model already evident from 65 °C

To assess whether a reduction in the amount of native whey proteins results in a loss of the allergy-protective capacity, milk samples were tested in a murine OVA-induced food allergy model. Mice were orally treated for eight consecutive days with the different heat-treated milk samples to determine whether the milk samples affected the development of OVA-induced food allergy. As expected, intradermal challenge with OVA increased the acute allergic skin response in OVA-sensitized allergic control mice compared to PBS-sensitized control mice (**Figure 5A**). Treating mice with raw milk prior to OVA-sensitization significantly reduced the acute allergic skin response compared to PBS-treated allergic control mice (**Figure 5A**). A shift in allergy protection appeared to occur from 60 to 65 °C; mice treated with milk heated at 60 °C still showed a significant reduction in the acute allergic skin response compared to PBS-treated allergic control mice, whereas milk heated at 65 °C was no longer protective (**Figure 5A**). A loss of allergy-protection was also observed in mice treated with milk heated at 70, 75, and 80 °C (**Figure 5A**). Although not significant, OVA-specific IgE levels were higher in OVA-sensitized allergic control mice compared to PBS-sensitized control mice (**Figure 5B**). No differences in OVA-IgE levels were observed between milk-treated mice (**Figure 5B**).

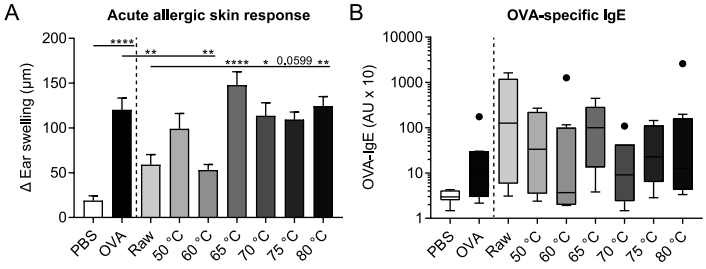


Figure 5. Heat treatment at 65 °C or higher destroyed allergy-protective capacity of raw milk in murine OVA-induced food allergy model. (**A**) The acute allergic skin response (expressed as Δ ear swelling) measured 1 h after intradermal challenge in both ear pinnae with OVA. (**B**) OVA-specific IgE levels measured in serum 16 h after oral challenge with OVA. Data are presented as mean ± SEM for the acute allergic skin response and as box-and-whisker Tukey plot (in which outliers are shown as separately plotted points) for OVA-specific IgE levels, $n = 6$ in PBS group and $n = 6$-8 in all other groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups (**A**) or Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups (**B**). OVA, ovalbumin; raw, raw cow's milk; 50-80 °C, raw cow's milk heated at the indicated temperature for 30 min; AU, arbitrary units.

Induction of regulatory Tr1 cells correspond to acute allergic skin response

Since regulatory T cells (Tregs) are key for the induction of oral tolerance to food proteins (41), the effect of the different milk types on Treg subsets in the spleen was assessed by flow cytometry. The percentage of $CD25^{+}FoxP3^{+}$ Treg cells was significantly decreased in OVA-sensitized allergic control mice compared to PBS-sensitized control mice (**Figure 6A**). $CD25^{+}FoxP3^{+}$ Treg cell frequency did, however, not differ between milk-treated mice (**Figure 6A**). Interestingly, the percentage of IL-10-producing Tr1 cells increased significantly in raw milk- and 50 °C milk-treated mice compared to PBS-treated allergic control mice (**Figure 6B**). This increase coincided with the reduced acute allergic skin response observed in these groups (**Figure 5A**). Although protection against allergic symptoms was also observed for milk heated at 60 °C, the percentage of Tr1 cells was not increased (**Figure 6B**). Compared to raw milk, milk heated at 65, 75 and 80 °C showed a reduction in Tr1 cell frequency (**Figure 6B**), which is in line with the observed loss of protection against the OVA-induced acute allergic skin response (**Figure 5A**). For the percentage of TGF-β-producing Th3 cells, the highest heating temperatures showed the lowest cell frequency, but differences were not significant (**Figure 6C**).

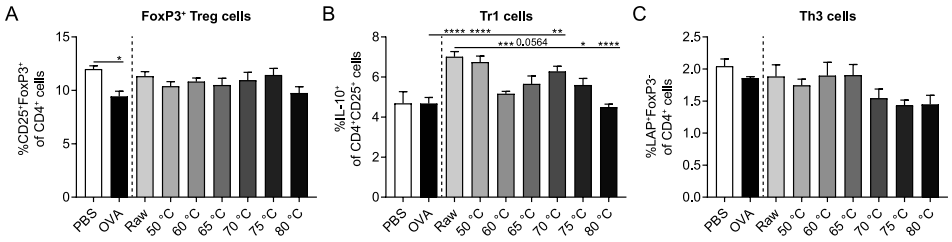


Figure 6. Percentage of regulatory Tr1 cells coincided with acute allergic skin response. Percentage of (**A**) FoxP3⁺ Treg cells (CD25⁺FoxP3⁺ of CD4⁺ cells), (**B**) Tr1 cells (IL-10⁺ of CD4⁺CD25⁺ cells), and (**C**) Th3 cells (LAP⁺FoxP3⁻ of CD4⁺ cells) in the spleen. Data are presented as mean ± SEM, n = 6 in PBS group and n = 6-8 in all other groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups. OVA, ovalbumin; raw, raw cow's milk; 50-80 °C, raw cow's milk heated at the indicated temperature for 30 min.

Milk heated at 70 °C or higher associated with increased percentages of Th1, Th2 and dendritic cells (DCs)

Besides Tregs, also other immune cells influence the strength of the allergic response. In order to investigate the effect of the different heating temperatures on Th1 cells, Th2 cells, and DCs, splenocytes were isolated and analyzed by flow cytometry. A decrease in Th1 cells was observed in OVA-sensitized allergic control mice compared to PBS-sensitized control mice (**Figure 7A**). Th2 and DC frequency did not differ between these control groups (**Figures 7B,C**). Although raw milk treatment protected against allergic symptoms, which was associated with an increased percentage of regulatory Tr1 cells, it also tended to increase the percentage of Th2 cells (**Figure 7B**). In addition, CD11c⁺MHCII⁺ DCs were elevated in raw milk-treated mice compared to PBS-treated allergic control mice (**Figure 7C**). Milk heated at 70 °C or higher increased the percentages of Th1, Th2, and DCs compared to PBS-treated allergic control mice (**Figures 7A-C**). These increased immune responses coincided with a loss of allergy protection (**Figure 5A**) and low numbers of regulatory Tr1 cells (**Figure 6B**).

Denaturation of several immunologically active whey proteins already started at 60/65 °C

Even though the overall protein profile (BCA assay, clustered heat map, and PCA) showed major differences in the native protein profile from 70/75 °C onwards, a loss of allergy protection was already demonstrated at 65 °C. To get a more detailed perspective on changes in immunologically active whey proteins, a heat map solely focusing on these specific whey proteins was created (**Figure 8**). In this heat map, a relatively clear difference is observed between milk samples heated at temperatures below 65 °C and milk samples heated at temperatures of 65 °C and above (**Figure 8**), which corresponds to the loss of allergy protection observed in the murine OVA-induced food allergy model (**Figure 5A**).

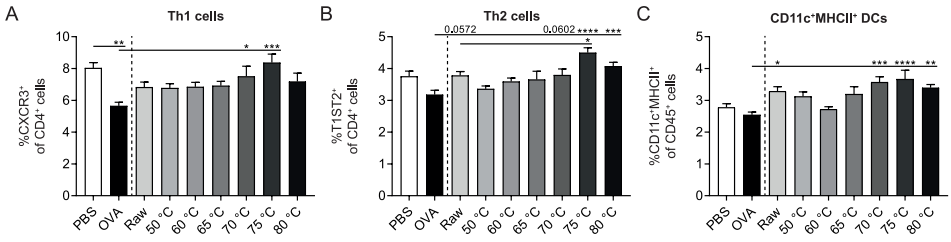


Figure 7. Increased Th1, Th2, and DC percentages at heating temperatures of 70 °C and above. Percentage of (**A**) Th1 cells (CXCR3$^+$ of CD4$^+$ cells), (**B**) Th2 cells (T1ST2$^+$ of CD4$^+$ cells), and (**C**) CD11c$^+$MHCII$^+$ DCs (CD11c$^+$MHCII$^+$ of CD45$^+$ cells) in the spleen. Data are presented as mean ± SEM, $n = 6$ in PBS group and $n = 6$-8 in all other groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups. OVA, ovalbumin; raw, raw cow's milk; 50-80 °C, raw cow's milk heated at the indicated temperature for 30 min.

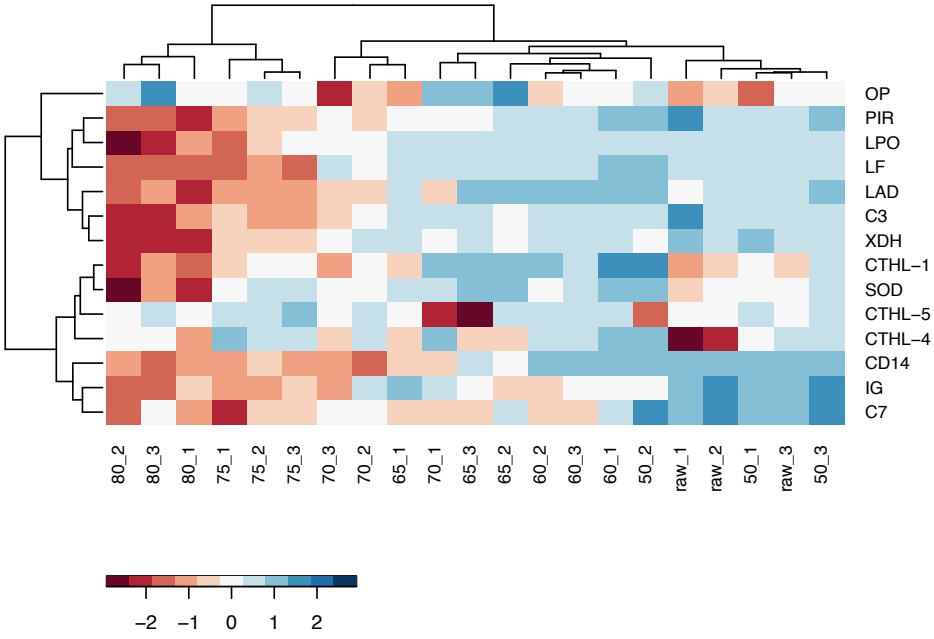


Figure 8. Hierarchical cluster analysis of immunologically active whey proteins demonstrated that the denaturation of these proteins already starts at 65 °C. Protein profile of heated milk serum samples based on Z score normalized iBAQ values. Columns reflect individual samples. The number indicates the heating temperature, while the number after the underscore indicates the replicate. Rows reflect immunologically active whey proteins. Hierarchical clustering was performed using complete agglomeration method and a Euclidean distance metric. The color scale reflects the iBAQ value-based Z score. OP, osteopontin; PIR, polymeric immunoglobulin receptor; LPO, lactoperoxidase; LF, lactoferrin; LAD, lactadherin; C3, complement component 3; XDH, xanthine dehydrogenase/oxidase; CTHL-1, cathelicidin-1; SOD, superoxide dismutase; CTHL-5, cathelicidin-5; CTHL-4, cathelicidin-4; CD14, monocyte differentiation antigen CD14; IG, immunoglobulin; C7, complement component 7.

Table 1. iBAQ values of immunologically active whey proteins after different heat treatments.

Log10 (iBAQ) / Protein name	Raw	50 °C	60 °C	65 °C	70 °C	75 °C	80 °C
Complement C7	3.86 ± 0.22	4.06 ± 0.14	2.96 ± 0.30*	2.95 ± 0.34*	2.99 ± 0.09*	2.38 ± 0.58*	2.38 ± 0.54*
Monocyte differentiation antigen CD14	5.02 ± 0.08	5.07 ± 0.20	4.98 ± 0.14	3.82 ± 0.69*	2.50 ± 0.44*	2.70 ± 0.18*	2.36 ± 0.28*
Polymeric immunoglobulin receptor	7.14 ± 0.61	6.92 ± 0.20	6.79 ± 0.11	6.03 ± 0.28*	5.64 ± 0.52*	4.99 ± 0.25*	3.96 ± 0.20*
Lactadherin	5.49 ± 0.10	5.85 ± 0.15	5.97 ± 0.07*	5.83 ± 0.19	4.93 ± 0.15*	4.62 ± 0.07*	4.30 ± 0.38*
Immunoglobulin	4.70 ± 0.15	4.29 ± 0.63	3.35 ± 0.35	3.71 ± 0.68	3.32 ± 0.99	2.54 ± 0.31*	2.12 ± 0.58*
Complement C3	5.90 ± 0.70	5.68 ± 0.09	5.71 ± 0.05	5.29 ± 0.36	5.03 ± 0.32	4.23 ± 0.38*	3.36 ± 0.74*
Lactoperoxidase	6.49 ± 0.15	6.54 ± 0.19	6.70 ± 0.08	6.61 ± 0.21	6.19 ± 0.25	4.74 ± 1.08*	3.49 ± 1.10*
Xanthine dehydrogenase/oxidase	5.40 ± 0.18	5.32 ± 0.33	5.37 ± 0.05	5.22 ± 0.26	4.98 ± 0.18	4.30 ± 0.22*	2.77 ± 0.19*
Lactoferrin	6.58 ± 0.02	6.61 ± 0.28	6.75 ± 0.11	6.44 ± 0.31	6.03 ± 0.23	2.85 ± 0.24*	2.16 ± 0.33*
Superoxide dismutase	4.28 ± 0.15	4.66 ± 0.19	4.68 ± 0.20	4.87 ± 0.16*	4.52 ± 0.21	4.59 ± 0.18	3.47 ± 0.40*
Cathelicidin-1	4.44 ± 0.13	4.90 ± 0.29	5.05 ± 0.15*	4.87 ± 0.30	4.70 ± 0.39	4.66 ± 0.09*	4.10 ± 0.26*
Cathelicidin-4	3.10 ± 0.77	4.04 ± 0.30*	4.09 ± 0.09*	3.55 ± 0.09	3.96 ± 0.46	4.12 ± 0.06*	3.65 ± 0.24
Cathelicidin-5	4.49 ± 0.14	4.29 ± 0.72	4.88 ± 0.07	4.08 ± 1.22	4.18 ± 0.92	4.89 ± 0.13	4.60 ± 0.07*
Osteopontin	6.54 ± 0.11	6.54 ± 0.25	6.61 ± 0.08	6.69 ± 0.30	6.49 ± 0.34	6.65 ± 0.04	6.79 ± 0.20*

Raw, raw cow's milk; 50-80 °C, raw cow's milk heated at the indicated temperature for 30 min. *$P < 0.05$ compared to raw milk as analyzed with Dunnett's multiple comparisons test.

To zoom in on the effects of the various heat treatments on the immunologically active whey proteins, and to get an idea about their concentration, **Table 1** was compiled to show the differences in iBAQ intensities for these proteins. Interestingly, complement component 7 (C7) decreased significantly at 60 °C and polymeric immunoglobulin receptor and monocyte differentiation antigen CD14 at 65 °C (**Table 1**). For immunoglobulins, denaturation also started already around 60/65 °C, but due to a larger variation the reduction only reached significance at 75 °C (**Table 1**). Most of the other immunologically active whey proteins only decreased significantly from 75 °C, although they already started to decrease, to varying extents, from lower temperatures (**Table 1**). For superoxide dismutase, cathelicidins, and osteopontin no clear decrease with increasing temperature was observed (**Table 1**).

DISCUSSION

The present study demonstrates that heat treatment of milk results in a substantial loss of native whey proteins due to denaturation and aggregation processes. A considerable decrease in the amount of native whey proteins was observed from 75 °C and above, but immunologically active whey proteins already started to decrease from 65 °C. Interestingly, the loss of immunologically active whey proteins coincided with the loss of allergy protection observed in a murine OVA-induced food allergy model. In this model, we observed a clear shift in allergy protection from 60 to 65 °C, where milk heated at 65 °C or higher was no longer protective.

Raw cow's milk consumption has repeatedly been reported to protect against childhood asthma and allergies (13-16, 21, 22), which has often been related to the bioactive whey protein fraction of the milk (18, 19), although a direct link between individual proteins and this protection has never been demonstrated. The whey protein fraction of raw cow's milk consists of hundreds of proteins and each of these proteins has its own denaturation kinetics (23). Heating milk at different temperatures may therefore result in different native protein profiles. Relating the changes in these native protein profiles to the capacity of the milk to prevent the development of allergic diseases may give an indication of the proteins responsible for the protective effect. The current study therefore used small differences in heating temperature (50, 60, 65, 70, 75, and 80 °C) and tested the various heat-treated milk samples for their native protein profile and their allergy-protective capacity.

As expected, the number of denatured proteins increased with increased heating intensity. Consequently, the native protein profile differed considerably between the different heat-treated milk samples. The overall protein data analyses (BCA assay, clustered heat map, and PCA) showed a significant difference between milk heated ≥ 75 °C and milk heated < 75 °C,

which is in line with our previous results (42). Similar effects were also reported by Brick *et al.* who demonstrated a decrease in detectable native whey proteins after heating with an intensity higher than pasteurization (23). According to the BCA assay, a significant decrease in the native whey protein concentration was also observed in milk heated at 70 °C. However, due to a larger variation within the triplicates, this group was not clustered with milk heated at 75 and 80 °C in the subsequent analyses. When specifically focusing on immunologically active whey proteins, their denaturation already started at a lower temperature. This is in accordance with previous studies, which demonstrated that particularly the immune active proteins present in the whey fraction have a high heat sensitivity (27). Although also for these proteins a sharp decrease was observed from 75 °C, a gradual decrease was already visible from 65 °C.

To determine the consequences of the heat-induced reduction in native whey proteins on the allergy-protective effects associated with raw milk, milk samples were tested in a murine OVA-induced food allergy model. Mice were orally treated for eight consecutive days with the different heat-treated milk samples and effects on the development of OVA-induced food allergy were subsequently assessed. As demonstrated before, treating mice with raw milk prior to sensitization and challenge with OVA significantly reduced the acute allergic skin response (22, 43). The current study shows that this protective effect lasted up to 60 °C, although it should be mentioned that the large variation in the group treated with milk heated at 50 °C prevented a significant effect. Interestingly, a loss of allergy protection was observed at heating temperatures of 65 °C and above, thereby coinciding with the denaturation of immunologically active whey proteins. The effects on the acute allergic skin response were not reflected in OVA-IgE levels but did correspond with regulatory Tr1 cell numbers, involved in the induction of oral tolerance to food proteins (41).

So even though major differences in the native protein profile were only observed from 70/75 °C onwards, a loss of allergy protection was already demonstrated at 65 °C. As mentioned earlier, immunologically active whey proteins were shown to denature from this temperature onwards. A closer look at the individual bioactive whey proteins revealed that the concentration of complement C7, monocyte differentiation antigen CD14, and polymeric immunoglobulin receptor decreased significantly at a heating temperature of 65 °C. Complement C7 is a protein involved in the complement system of the innate immune system and is part of the membrane attack complex. How complement C7 may be involved in the allergy-protective effect of raw cow's milk remains unclear, although a role of the complement pathway in the development of asthma and allergy has been described (44). Monocyte differentiation antigen CD14 is also a molecule of the innate immune system and acts as a receptor and carrier for bacterial endotoxin. Interestingly, CD14 expression was markedly higher in farmers' children than in non-farmers' children (45). In addition, polymorphisms in the CD14 gene have been

demonstrated to modify the effect of raw cow's milk consumption on allergic diseases (46, 47). Polymeric immunoglobulin receptor is an Fc receptor which facilitates the transcytosis of soluble IgA and immune complexes. Breast milk immune complexes are shown to be potent inducers of oral tolerance and prevented asthma development in mice (48). Whether the formation of immune complexes also contributes to the allergy-protective effect of raw cow's milk remains to be elucidated.

The significant decrease in complement C7, CD14, and polymeric immunoglobulin receptor concentrations after heat treatment at 65 °C may partly explain the loss of allergy protection at the same temperature. However, many other immunologically active whey proteins also showed a, non-significant, decrease around 65 °C. Because of the many immunologically active whey proteins present in raw cow's milk, even small changes in each of them could affect the final allergic response. In addition, we have to acknowledge that proteins like enzymes, with a relatively low abundance but a high activity, such as alkaline phosphatase, were not detected with the method used. The wide variety of proteins and the subtle changes in each of them after the different heat treatments makes it hard to pinpoint one unique protein responsible for the allergy-protective effect. Eventually, the synergistic effect of changes in several proteins simultaneously probably underlies the loss of protection.

In addition to the observed loss of protection from 65 °C, this study also demonstrates that raw milk can be heated up to 60 °C (for 30 min) without negative consequences for its allergy-protective capacity. Immunologically active whey proteins were hardly affected by these temperatures and although milk heated at 50 °C did not significantly reduce the acute allergic skin response, the PCA could not distinguish between 50- and 60 °C-treated milk samples. This knowledge can be very relevant for the development of mildly processed milk in which bioactive raw milk components are retained.

Besides the loss of immunologically active whey proteins, the formation of protein aggregates can also underlie the loss of allergy protection upon heat treatment. These protein aggregates were reported to have an increased immunogenicity compared to their native counterparts because of a shifted uptake from enterocytes to Peyer's patches (28). Heat-induced protein aggregate formation, through disulphide interchange, was therefore assessed in the differentially heated milk samples. Protein aggregates started to form from 75 °C onwards and might therefore be responsible for the enhanced immune response, demonstrated by increased percentages of Th1 cells, Th2 cells, and DCs, observed in mice treated with milk samples heated at the same temperature. However, since the loss of allergy protection was already demonstrated from 65 °C, the formation of immunogenic protein aggregates is most likely not responsible for this effect.

In conclusion, we demonstrated that the allergy-protective effect of raw cow's milk is lost after heating milk for 30 min at 65 °C or higher. This loss of protection coincided with a reduction in native immunologically active whey proteins. A significant reduction was demonstrated for complement C7, monocyte differentiation antigen CD14, and polymeric immunoglobulin receptor, but many other immunologically active whey proteins also showed a decrease around 65 °C. As, possible immunogenic, aggregates were not yet formed at this temperature, they probably do not play a role in the allergy-protective effect of raw cow's milk. The current study thereby provides a better understanding of the mechanistic relation between heat damage to whey proteins and allergy development, which is essential for the development of microbiologically safe alternatives to raw cow's milk.

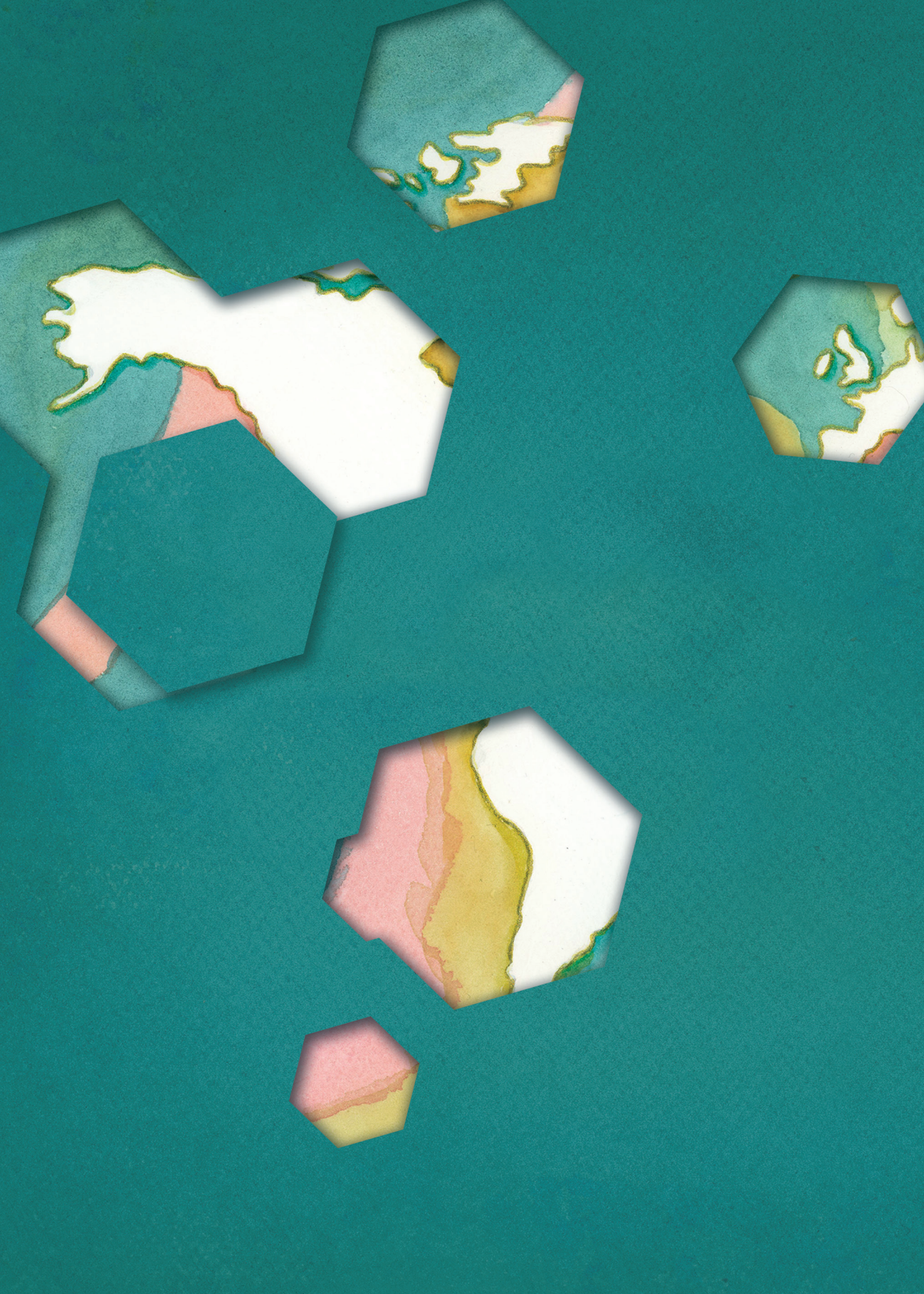
REFERENCES

1. European Academy of Allergy and Clinical Immunology. Global atlas of allergy. (2014) Available from: http://www.eaaci.org/GlobalAtlas/GlobalAtlasAllergy.pdf Accessed on: 12 June 2019
2. Eder W, Ege MJ, and von Mutius E. The asthma epidemic. *N Engl J Med* (2006) 355(21): 2226-35.
3. Alfvén T, Braun-Fahrlander C, Brunekreef B, von Mutius E, Riedler J, Scheynius A, *et al*. Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle - The PARSIFAL study. *Allergy* (2006) 61(4): 414-21.
4. Braun-Fahrlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS, *et al*. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. *Clin Exp Allergy* (1999) 29(1): 28-34.
5. Kilpelainen M, Terho EO, Helenius H, and Koskenvuo M. Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* (2000) 30(2): 201-8.
6. Riedler J, Eder W, Oberfeld G, and Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin Exp Allergy* (2000) 30(2): 194-200.
7. von Ehrenstein OS, von Mutius E, Illi S, Baumann L, Bohm O, and von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* (2000) 30(2): 187-93.
8. Ernst P and Cormier Y. Relative scarcity of asthma and atopy among rural adolescents raised on a farm. *Am J Respir Crit Care Med* (2000) 161(5): 1563-6.
9. von Mutius E and Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* (2010) 10(12): 861-8.
10. Remes ST, Iivanainen K, Koskela H, and Pekkanen J. Which factors explain the lower prevalence of atopy amongst farmers' children? *Clin Exp Allergy* (2003) 33(4): 427-34.
11. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrlander C, *et al*. Exposure to environmental microorganisms and childhood asthma. *N Engl J Med* (2011) 364(8): 701-9.
12. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
13. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
14. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
15. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
16. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-706 e13.
17. Committee on Infectious Diseases & Committee on Nutrition & American Academy of Pediatrics. Consumption of raw or unpasteurized milk and milk products by pregnant women and children. *Pediatrics* (2014) 133(1): 175-9.
18. van Neerven RJ, Knol EF, Heck JM, and

Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
19. Abbring S, Hols G, Garssen J, and van Esch BCAM. Raw cow's milk consumption and allergic diseases - The potential role of bioactive whey proteins. *Eur J Pharmacol* (2019) 843: 55-65.
20. Braun-Fahrländer C and von Mutius E. Can farm milk consumption prevent allergic diseases? *Clin Exp Allergy* (2011) 41(1): 29-35.
21. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T*, et al*. Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model. *Front Immunol* (2017) 8: 1045.
22. Abbring S, Ryan JT, Diks MAP, Hols G, Garssen J, and van Esch BCAM. Suppression of food allergic symptoms by raw cow's milk in mice is retained after skimming but abolished after heating the milk - A promising contribution of alkaline phosphatase. *Nutrients* (2019) 11(7): 1499.
23. Brick T, Ege M, Boeren S, Bock A, von Mutius E, Vervoort J*, et al*. Effect of processing intensity on immunologically active bovine milk serum proteins. *Nutrients* (2017) 9(9): 963.
24. Li-Chan E, Kummer A, Losso JN, Kitts DD, and Nakai S. Stability of bovine immunoglobulins to thermal-treatment and processing. *Food Res Int* (1995) 28(1): 9-16.
25. Chen WL, Hwang MT, Liau CY, Ho JC, Hong KC, and Mao SJT. Beta-Lactoglobulin is a thermal marker in processed milk as studied by electrophoresis and circular dichroic spectra. *J Dairy Sci* (2005) 88(5): 1618-30.
26. McGuffey MK, Epting KL, Kelly RM, and Foegeding EA. Denaturation and aggregation of three alpha-lactalbumin preparations at neutral pH. *J Agr Food Chem* (2005) 53(8): 3182-90.
27. Zhang LN, Boeren S, Smits M, van Hooijdonk T, Vervoort J, and Hettinga K. Proteomic study on the stability of proteins in bovine, camel, and caprine milk sera after processing. *Food Res Int* (2016) 82: 104-11.
28. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J*, et al*. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy* (2008) 63(7): 882-90.
29. Bundesministerium der Justiz und für Verbraucherschutz. Verordnung über die Güteprüfung und Bezahlung der Anlieferungsmilch (Milch-Güteverordnung). (1980) Available from: http://www.gesetze-im-internet.de/milchg_v/index.html Accessed on: January 9 2019
30. Law AJ and Leaver J. Effect of pH on the thermal denaturation of whey proteins in milk. *J Agric Food Chem* (2000) 48(3): 672-9.
31. Wiśniewski JR, Zougman A, Nagaraj N, and Mann M. Universal sample preparation method for proteome analysis. *Nat Methods* (2009) 6(5): 359.
32. Hettinga K, Van Valenberg H, De Vries S, Boeren S, Van Hooijdonk T, van Arendonk J*, et al*. The host defense proteome of human and bovine milk. *PloS one* (2011) 6(4): e19433.
33. Lu J, Boeren S, De Vries S, Van Valenberg H, Vervoort J, and Hettinga K. Filter-aided sample preparation with dimethyl labeling to identify and quantify milk fat globule membrane proteins. *J Proteomics* (2011) 75(1): 34-43.
34. Zhang L, Boeren S, Hageman JA, van Hooijdonk T, Vervoort J, and Hettinga K. Bovine milk proteome in the first 9 days: protein interactions in maturation of the immune and digestive system of the newborn. *PLoS One* (2015) 10(2): e0116710.
35. Zhang L, Boeren S, Hageman JA, van Hooijdonk T, Vervoort J, and Hettinga K. Perspective on calf and mammary gland development through changes in the bovine milk proteome over a complete lactation. *J Dairy Sci* (2015) 98(8): 5362-73.
36. Cox J and Mann M. MaxQuant enables high peptide identification rates, individualized

p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* (2008) 26: 1367.

37. UniProt C. Universal protein resource (Uniprot). (2013) Available from: http://www.uniprot.org/ Accessed on: March 2019
38. Schwanhausser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, *et al*. Global quantification of mammalian gene expression control. *Nature* (2011) 473(7347): 337-42.
39. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, *et al*. The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* (2016) 13(9): 731-40.
40. The R Development Core Team. R: a language and environment for statistical computing. (2013) Available from: https://www.R-project.org/ Accessed on: March 2019
41. Pabst O and Mowat AM. Oral tolerance to food protein. *Mucosal Immunol* (2012) 5(3): 232-9.
42. Xiong L, Li C, Boeren S, Vervoort J, and Hettinga K. Effect of heat treatment on the proteome composition and the bacteriostatic activity of bovine milk serum. *Under review* (2019).
43. Abbring S, Wolf J, Ayechu Muruzabal V, Diks MAP, Alashkar Alhamwe B, Alhamdan F, *et al*. Raw cow's milk reduces allergic symptoms in a murine model for food allergy - A potential role for epigenetic modifications. *Nutrients* (2019) 11(8): 1721.
44. Gerard NP and Gerard C. Complement in allergy and asthma. *Curr Opin Immunol* (2002) 14(6): 705-8.
45. Lauener RP, Birchler T, Adamski J, Braun-Fahrlander C, Bufe A, Herz U, *et al*. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. *Lancet* (2002) 360(9331): 465-6.
46. Bieli C, Eder W, Frei R, Braun-Fahrlander C, Klimecki W, Waser M, *et al*. A polymorphism in CD14 modifies the effect of farm milk consumption on allergic diseases and CD14 gene expression. *J Allergy Clin Immunol* (2007) 120(6): 1308-15.
47. Ege MJ, Strachan DP, Cookson WO, Moffatt MF, Gut I, Lathrop M, *et al*. Gene-environment interaction for childhood asthma and exposure to farming in Central Europe. *J Allergy Clin Immunol* (2011) 127(1): 138-44.
48. Mosconi E, Rekima A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, *et al*. Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. *Mucosal Immunol* (2010) 3(5): 461-74.



CHAPTER 7

Spiking processed milk with bioactive whey proteins as alternative to raw cow's milk – A preliminary study

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ABSTRACT

The consumption of raw cow's milk as an allergy-preventive measure is promising, but the risk of infections hampers usability. As an alternative to raw milk, specific raw milk components for supplementing processed milk may be interesting. Since bioactive whey proteins are often implied to be responsible for the allergy-preventive potential of raw cow's milk, the present study assessed whether alkaline phosphatase (ALP), lactoferrin (LF), osteopontin (OPN), and IgG could restore the allergy-protective effect lost upon milk processing. Female C3H/HeOuJ were orally treated with PBS, raw milk, processed milk, processed milk spiked with ALP, with LF, with OPN, with IgG, or with a combination of these four whey proteins for eight consecutive days prior to sensitization and challenge with ovalbumin (OVA). Effects on acute allergic symptoms, basophil activation, and immunoglobulin levels were subsequently assessed. Raw milk-treated mice showed a reduced OVA-induced allergic response compared to processed milk-treated mice. Of the four individual whey proteins tested, only the addition of LF to processed milk was able to reduce the acute allergic skin response compared to processed milk alone, although all four whey proteins reduced anaphylactic shock symptoms. OPN-treated mice furthermore showed a reduction in both basal and OVA-specific basophil activation. Spiking processed milk with the combination of ALP, LF, OPN, and IgG reduced the acute allergic skin response, anaphylactic shock symptoms, and basophil activation compared to processed milk alone. In addition, a tendency towards a reduction in OVA-IgE levels was observed in this group. Due to the lack of a strong allergic response in the positive control group, the obtained results require careful interpretation. Processed milk spiked with the combination of the four bioactive whey proteins most closely resembled the allergy-protective raw milk effect. Of the individual whey proteins tested, LF was most effective in restoring the allergy-protective effect lost upon milk processing, although ALP, OPN, and IgG also showed promising effects. This study thereby provides preliminary evidence that spiking processed milk with bioactive whey proteins, as an alternative to raw cow's milk, is a promising preventive approach for allergic diseases.

INTRODUCTION

Today, the consumption of raw, unprocessed, cow's milk is much less common than in the past. The potential risk of bacterial infections that can be transmitted by raw milk when contaminated with human pathogens is reason for regulatory authorities to prohibit or strongly discourage its consumption (1). Nevertheless, raw cow's milk is still consumed by a considerable number of people. Particularly farming families, but to some extent also rural, non-farming, families often prefer to drink milk directly from the cow (2).

Epidemiological studies among these families have repeatedly shown that the consumption of raw cow's milk is associated with a reduced risk of developing childhood asthma and allergy (3-8). These observed associations were recently strengthened by causality. In preclinical animal models, raw cow's milk prevented the development of house dust mite-induced allergic asthma and of ovalbumin (OVA)-induced food allergy (9-11). Interestingly, both the epidemiological and the preclinical studies have demonstrated a loss of protection upon milk processing. In contrast to raw milk, boiled raw milk and commercially available shop milk were not able to reduce allergy risk (7-10). These results indicate that although industrial milk processing techniques solve the critical issue of milk-borne infections, they also induce unwanted changes in the milk composition thereby destroying the allergy-protective capacity of the milk. Milder processing to retain the bioactive raw milk components or the addition of these components to heat-treated milk might be interesting alternatives but require clarification of the raw milk components involved in the allergy-protective effects.

Basically all components affected by industrial milk processing are candidates for the asthma- and allergy-protective potential of raw cow's milk. As a result, a wide variety of components are hypothesized to contribute to the observed effects (2, 12, 13). However, since recent research demonstrates that the suppression of food allergic symptoms by raw milk is retained after skimming but abolished after pasteurization of the milk (10), heat-sensitive raw milk components are the most likely constituents to be involved. Of these heat-sensitive raw milk components, particularly the whey protein fraction seems to be of interest since many of the proteins present in this fraction have immune-related functionalities that can be linked to the allergy-protective effects of raw cow's milk (12, 13). Previously, we already showed the potential of this bioactive whey protein fraction by demonstrating that native whey proteins have a lower allergenicity than their processed counterparts (14). Whether the native whey protein fraction is also responsible for the allergy-preventive feature of raw cow's milk remains to be elucidated.

With the current research, we aimed to assess whether the bioactive whey proteins alkaline phosphatase (ALP), lactoferrin (LF), osteopontin (OPN), and IgG have the capacity to restore the allergy-protective effect lost upon milk processing. Mice were treated for eight consecutive days with raw milk, processed milk, processed milk spiked with ALP, with LF, with OPN, or with IgG and effects on OVA-induced allergic symptoms were examined. In addition, the four immunologically active whey proteins were combined to investigate whether this results in a synergistic effect.

MATERIALS & METHODS

Animals

Three-week-old, specific pathogen-free, female C3H/HeOuJ mice (The Jackson Laboratory, Bar Harbor, ME, USA) were housed at the animal facility of Utrecht University (Utrecht, The Netherlands) in filter-topped makrolon cages (one cage/group, *n* = 6-8/cage) with standard chip bedding, Kleenex tissues, and a plastic shelter, on a 12 h light/dark cycle with access to food ('Rat and Mouse Breeder and Grower Expanded'; Special Diet Services, Witham, UK) and water *ad libitum*. Upon arrival, mice were randomly assigned to the control and experimental groups and were habituated to the laboratory conditions for six days prior to the start of the study. All animal procedures were approved by the Ethical Committee for Animal Research of the Utrecht University and were complied with the European Directive 2010/63/EU on the protection of animals used for scientific purposes (AVD108002015346).

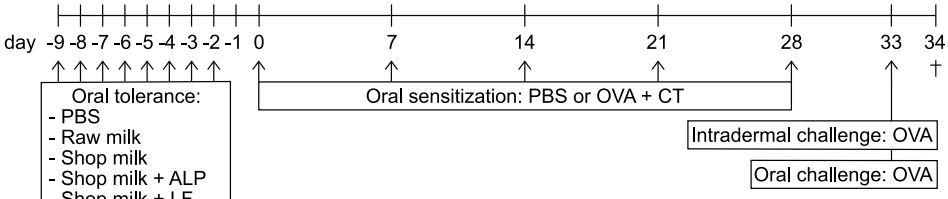
Milk types and bioactive whey proteins

Raw, unprocessed, cow's milk was collected from a biodynamic dairy farm legally allowed to sell raw milk (organic 'Vorzugsmilch' (15); Hof Dannwisch, Horst, Germany). Shop milk (full fat, store-bought, milk) was obtained from Melkan Superunie (Beesd, The Netherlands). On the days of milk treatment (experimental days -9 to -2; **Figure 1**), part of the shop milk was spiked with the bioactive whey proteins ALP, LF, OPN, IgG, or a combination of these four. Bovine intestinal ALP (1.5 units/0.5 mL shop milk) was kindly provided by prof. dr. W. Seinen (Alloksys Life Sciences BV/AMRIF BV, Wageningen, The Netherlands) and bovine milk OPN (0.05 mg/0.5 mL shop milk) by Arla Foods (Viby, Denmark). Bovine milk LF (0.325 mg/0.5 mL shop milk) was obtained from Synlait Milk (Canterburg, New Zealand) and bovine serum IgG (0.63 mg/0.5 mL shop milk) from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Experimental design – Tolerance induction, sensitization and challenges

On experimental days 0, 7, 14, 21, and 28, mice (*n* = 8/group) were orally sensitized, by using a blunt needle, to the hen's egg protein OVA (20 mg/0.5 mL PBS; grade V; Sigma-Aldrich) using cholera toxin (CT; 15 µg/0.5 mL; List Biological Laboratories, Campbell, CA, USA) as an adjuvant. Sham-sensitized control mice (*n* = 6) received CT alone (15 µg/0.5

mL PBS). To remove lipopolysaccharide, OVA solutions were passed through Pierce High Capacity Endotoxin Removal Resin (Thermo Fisher Scientific, Paisley, Scotland) prior to use. Prior to sensitization, mice were orally treated with 0.5 mL PBS (as a control), raw milk, shop milk, shop milk spiked with ALP, with LF, with OPN, with IgG, or with a combination of these four bioactive whey proteins for eight consecutive days (days -9 to -2). On day 27, one day before the last sensitization, a blood sample was taken via cheek puncture to determine basophil activation. On day 33, five days after the last sensitization, all mice were challenged intradermally in both ear pinnae with OVA (10 µg/20 µL PBS) to determine the acute allergic response. On the same day (approximately 7 h after the intradermal challenge), mice received an oral challenge (50 mg OVA/0.5 mL PBS). Sixteen hours later (day 34), mice were killed by cervical dislocation and blood was collected. A schematic overview of the experimental timeline is shown in **Figure 1**.



Group	*n*	Tolerance	Sensitization	Challenge
PBS	6	PBS	PBS + CT	OVA
OVA	8	PBS	OVA + CT	OVA
Raw milk	8	Raw milk	OVA + CT	OVA
Shop milk	8	Shop milk	OVA + CT	OVA
Shop milk + ALP	8	Shop milk + ALP	OVA + CT	OVA
Shop milk + LF	8	Shop milk + LF	OVA + CT	OVA
Shop milk + OPN	8	Shop milk + OPN	OVA + CT	OVA
Shop milk + IgG	8	Shop milk + IgG	OVA + CT	OVA
Shop milk + combi	8	Shop milk + combi	OVA + CT	OVA

Figure 1. Schematic overview of the study design. Female C3H/HeOuJ mice were orally treated with 0.5 mL PBS (as a control), raw milk, shop milk, shop milk spiked with ALP, with LF, with OPN, with IgG, or with a combination of these four bioactive whey proteins for eight consecutive days (experimental days -9 to -2). Following this tolerance induction period, mice were orally sensitized (on days 0, 7, 14, 21, and 28) to the hen's egg protein OVA using CT as an adjuvant (20 mg OVA + 15 µg CT/0.5 mL PBS). PBS-sensitized control mice received CT alone. Subsequently, all mice were intradermally (10 µg OVA/20 µL PBS) and orally (50 mg OVA/0.5 mL PBS) challenged with OVA (day 33). Sixteen hours after the oral challenge (day 34), mice were killed by cervical dislocation (as indicated by †). ALP, alkaline phosphatase; LF, lactoferrin; OPN, osteopontin; combi, combination of the four bioactive whey proteins ALP, LF, OPN, and IgG; OVA, ovalbumin; CT, cholera toxin.

Evaluation of the acute allergic response

To assess the severity of the acute allergic symptoms upon intradermal challenge in both ear pinnae with OVA, the acute allergic skin response, anaphylactic shock symptoms, and body temperature were evaluated by a researcher blinded to treatment. The acute allergic skin response, expressed as Δ ear swelling (μm), was calculated by subtracting the mean basal ear thickness from the mean ear thickness measured 1 h after intradermal challenge. Ear thickness at both timepoints was measured in duplicate for each ear using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). To perform the intradermal challenge and both ear measurements, mice were anesthetized using inhalation of isoflurane (Abbott, Breda, The Netherlands). The severity of the anaphylactic shock symptoms was scored 45 min after the intradermal challenge by using a previously described, validated, scoring table (16). To monitor the anaphylactic shock-induced drop in body temperature, the body temperature was also measured 45 min after intradermal challenge by using a rectal thermometer.

Assessment of basophil activation

Whole blood was drawn from each mouse via cheek puncture on day 27. The basophil activation test (BAT) was subsequently performed as described previously by Torrero *et al.* (17). Briefly, blood samples from two mice were pooled and incubated with RPMI 1640 medium (Lonza, Verviers, Belgium), anti-mouse IgE (0.125 μg/mL; eBioscience, Breda, The Netherlands) or OVA (20 μg/mL; Sigma-Aldrich) for 90 min at 37 °C. Activation was stopped by adding PBS containing 5 mM EDTA (Thermo Fisher Scientific). Red blood cells were then lysed, and samples were fixed using a whole blood lysing reagent kit (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. To block non-specific binding sites, cells were subsequently incubated for 15 min on ice with anti-mouse CD16/CD32 (Mouse BD Fc Block; BD Biosciences, Alphen aan de Rijn, The Netherlands). Thereafter, cells were stained for 30 min on ice with CD4-PE, CD45R/B220-PE, IgE-FITC, and CD49b-APC (all from eBioscience) to select basophils, while excluding T cells and B cells. Median fluorescence intensity (MFI) of CD200R-PerCP-eFluor® 710 (eBioscience) was used to determine basophil activation. Flow cytometry was performed using FACS Canto II (BD Biosciences) and the results were analyzed using FlowLogic Software (Inivai Technologies, Mentone, Australia). Isotype controls were used for each antibody and cut-off gates for positivity were determined with fluorescence-minus-one controls.

Measurement of serum OVA-specific IgE and IgG1

Blood collected via cheek puncture prior to sacrifice (day 34) was centrifuged at 10,000 rpm for 10 min. Serum was subsequently obtained and stored at -20 °C until analysis of OVA-specifc IgE and IgG levels by means of ELISA as described elsewhere (10).

Statistical analysis

Experimental results are expressed as mean ± SEM or as individual data points or box-and-whisker Tukey plots when data were not normally distributed. In addition, individual data points are displayed in the control groups for acute allergic symptoms. Differences between pre-selected groups were statistically determined using one-way ANOVA, followed by Bonferroni's multiple comparisons test. OVA-specific IgE and IgG1 levels were log- and square root-transformed, respectively, prior to ANOVA analysis. Anaphylactic shock scores were analyzed using Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups. Statistical analyses were performed using GraphPad Prism software (version 7.03; GraphPad Software, San Diego, CA, USA) and results were considered statistically significant when $P < 0.05$.

RESULTS

Addition of bioactive whey proteins to heat-treated milk suppresses OVA-induced allergic symptoms

Previously, we demonstrated the capacity of raw cow's milk to prevent the development of OVA-induced food allergy in a murine animal model. This protective effect was lost once the milk was heat-treated (10). To gain more insight into the heat-sensitive raw milk components involved in the allergy-protective effect, a selected set of bioactive whey proteins (ALP, LF, OPN, IgG, or a combination of these four) was added to a processed, shop milk to assess whether they could restore the protective effect. Mice were orally treated for eight consecutive days with these spiked milk samples before being sensitized and challenged with OVA. Unexpectedly, and in contrast to many of our previous studies (10, 11, 18), intradermal challenge with OVA did not increase acute allergic symptoms in OVA-sensitized allergic mice compared to PBS-sensitized control mice (**Figures 2A-C**). Despite the lack of a strong allergic response in the positive control group, shop-milk treated mice showed an increased acute allergic skin response, increased anaphylactic shock symptoms and an anaphylactic shock-induced drop in body temperature after intradermal challenge with OVA compared to raw milk-treated mice (**Figures 2A-C**). Spiking shop milk with LF or the combination of the four bioactive whey proteins significantly reduced the acute allergic skin response compared to shop milk alone (**Figure 2A**). All bioactive whey proteins furthermore lowered anaphylactic shock symptoms whereas none of them affected body temperature (**Figures 2B,C**).

Milk treatments influence basophil activation

The acute allergic response measured upon intradermal challenge with OVA is primarily elicited by mast cell activation. Besides mast cells, also basophils are important effector cells in the allergic response. To determine basophil activation, whole blood was stimulated with

medium, OVA, or αIgE, and CD200R surface expression was assessed. Interestingly, baseline basophil activation (medium stimulation) already differed between groups. Shop milk-treated mice showed a significant higher CD200R expression on the surface of their basophils than raw milk-treated mice (**Figure 3A**). CD200R expression was furthermore reduced on basophils of mice treated with shop milk + OPN compared to mice treated with shop milk alone (**Figure 3A**). A similar reduction was observed after supplementing shop milk with the combination of the four bioactive whey proteins (**Figure 3A**). OVA-specific basophil activation only tended to decrease (P = 0.0523) in the OPN-spiked milk group compared to the shop milk group (**Figure 3B**). No differences between groups were observed after stimulation of whole blood with αIgE (**Figure 3C**).

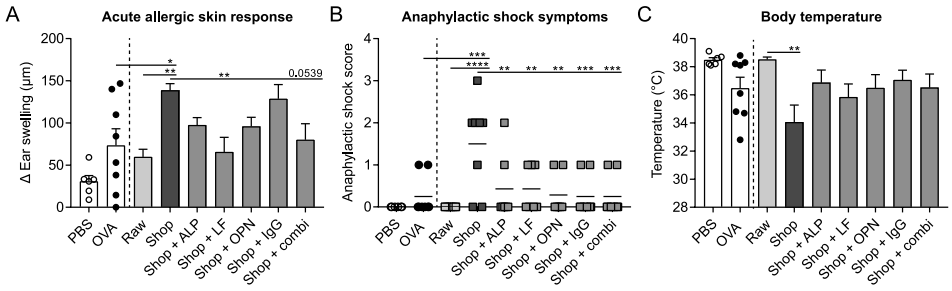


Figure 2. Reduced acute allergic symptoms upon OVA challenge in mice treated with processed milk supplemented with bioactive whey proteins. (**A**) The acute allergic skin response, expressed as Δ ear swelling, measured 1 h after intradermal challenge in both ear pinnae with OVA. (**B**) Anaphylactic shock scores and (**C**) body temperature determined 45 min after intradermal challenge. Data are presented as mean ± SEM for the acute allergic skin response and body temperature (with individual data points displayed in the control groups) and as individual data points for anaphylactic shock scores, n = 6 in PBS group and n = 7-8 in all other groups. Significant outliers were excluded based on the Grubbs' test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups (**A, C**) or Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups (**B**). OVA, ovalbumin; raw, raw cow's milk; shop, processed shop milk; ALP, alkaline phosphatase; LF, lactoferrin; OPN, osteopontin; combi, combination of the four bioactive whey proteins ALP, LF, OPN, and IgG.

Low OVA-specific IgE levels in serum of mice treated with spiked milk samples

Since type I hypersensitivity reactions, as often induced by food allergens, are characterized by the production of allergen-specific IgE antibodies, serum OVA-IgE levels were determined. Although not significant, OVA-specific IgE levels were higher in OVA-sensitized allergic mice compared to PBS-sensitized control mice (**Figure 4A**). OVA-IgE levels in the raw milk and shop milk group corresponded to the effects observed on acute allergic symptoms and basophil activation, with low levels in the raw milk group and higher levels in the shop milk group (**Figure 4A**). Low OVA-IgE levels were also observed in the milk groups spiked with individual

whey proteins, but differences did not reach significance (**Figure 4A**). Interestingly, adding the four bioactive whey proteins together to shop milk tended to decrease OVA-IgE levels (P = 0.0706) compared to shop milk alone (**Figure 4A**). OVA-IgG1 levels followed a similar pattern as OVA-IgE but did not differ between groups (**Figure 4B**).

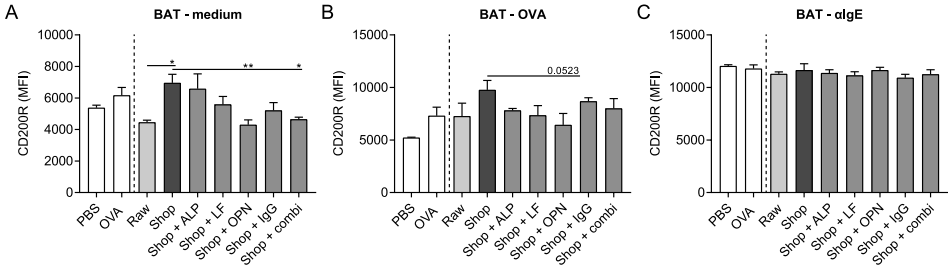


Figure 3. Basophil activation affected by milk treatments. Basophil activation determined at day 27 by surface expression of CD200R upon stimulation of whole blood with (**A**) medium, (**B**) OVA, or (**C**) αIgE. Blood of two mice were pooled. Data are presented as mean ± SEM, n = 3 in PBS group and n = 3-4 in all other groups. *P < 0.05, **P < 0.01, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups. BAT, basophil activation test; OVA, ovalbumin; raw, raw cow's milk; shop, processed shop milk; ALP, alkaline phosphatase; LF, lactoferrin; OPN, osteopontin; combi, combination of the four bioactive whey proteins ALP, LF, OPN, and IgG; MFI, median fluorescence intensity.

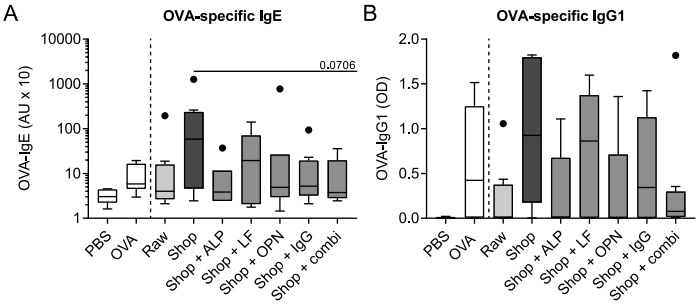


Figure 4. Serum OVA-specific IgE levels were low in mice treated with spiked milk samples. (**A**) Serum OVA-specific IgE and (**B**) IgG1 antibody levels measured 16 h after oral challenge. Data are presented as box-and-whisker Tukey plots (in which outliers are shown as separately plotted points), n = 6 in PBS group and n = 7-8 in all other groups, and analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups. OVA, ovalbumin; raw, raw cow's milk; shop, processed shop milk; ALP, alkaline phosphatase; LF, lactoferrin; OPN, osteopontin; combi, combination of the four bioactive whey proteins ALP, LF, OPN, and IgG; AU, arbitrary units; OD, optical density.

DISCUSSION

There is an increasing body of evidence demonstrating that the consumption of raw, unprocessed, cow's milk confers protection against allergic diseases (3-11). However, since raw milk may contain pathogens when not produced under strict hygienic and microbiological standards, its consumption cannot be recommended, and safe and allergy-protective alternatives need to be developed. To be able to develop these alternatives, a better understanding of the raw milk component(s) involved in the allergy-protective effect is essential. Previously, we demonstrated that particularly heat treatment is detrimental to the protective effects and we speculated about the potential contribution of the heat-sensitive whey proteins (10). In the present study, we therefore assessed the ability of a selected set of these whey proteins (ALP, LF, OPN, and IgG) to restore the allergy-protective effect lost after milk processing.

In this study, we demonstrate that spiking processed milk with bioactive whey proteins has great potential as alternative to raw cow's milk. Adding the combination of ALP, LF, OPN, and IgG to a processed, shop, milk most effectively reduced the OVA-induced allergic response, as demonstrated by reduced acute allergic symptoms, reduced basophil activation and a tendency towards lower OVA-specific IgE levels compared to shop milk. Shop milk spiked with the combination of the four bioactive whey proteins thereby most closely resembled the allergy-protective raw milk effect. Of the individual whey proteins, LF was most effective in restoring the allergy-protective effect lost upon milk processing, although ALP, OPN, and IgG also showed promising effects. The lack of a strong allergic response in the positive control group requires careful interpretation of the obtained results but does not diminish their relevance.

The whey protein fraction of raw cow's milk consists of a multitude of proteins and many of them have immune-related functionalities (19). Four of these immunologically active whey proteins, ALP, LF, OPN, and IgG, are often mentioned in relation to the allergy-protective effects of raw cow's milk (7, 12, 13) and were therefore selected for the current study. The four bioactive whey proteins were furthermore also combined as interactions between them have been described and might result in synergistic effects.

Of the individual bioactive whey proteins, the strongest reduction in allergic symptoms was observed with LF. When added to shop milk, LF significantly reduced the acute allergic skin response and anaphylactic shock symptoms compared to shop milk alone. LF is an iron-binding protein that belongs to the family of antimicrobial molecules (20). Besides antimicrobial properties, LF also has immunomodulatory capacities that might contribute to the observed allergy-protective effect. LF, for example, promotes the growth of bacteria with low iron requirements such as *Bifidobacteria* and *Lactobacilli* (21-23). These bacteria are considered to

be beneficial to the host and their presence in the gut microbiota of infants seems to correlate with protection against allergic diseases (24, 25). In addition, LF stimulates the production of TGF-β and IL-10 in the gut (26-28). Since both of these anti-inflammatory cytokines have a strong tolerogenic capacity (29, 30), lactoferrin supplementation might create a regulatory environment which favors unresponsiveness upon allergen exposure.

ALP is probably best known for its role in dairy industry as indicator of successful pasteurization (31). However, ALP also has many biological functions, of which the dephosphorylation-mediated detoxification of bacterial LPS is an example (32). This feature underlies the capacity of orally administered ALP to ameliorate inflammatory diseases, such as necrotizing enterocolitis, sepsis, and inflammatory bowel disease (33-35). Interestingly, we previously demonstrated that ALP can also affect allergic diseases. ALP fully restored the allergy-protective effect abolished by heat treatment in a murine OVA-induced food allergy model (10). In the current study, a lower concentration of ALP (1.5 units/0.5 mL shop milk instead of 3 units/0.5 mL) again showed promising effects, as demonstrated by reduced anaphylactic shock symptoms upon OVA challenge. However, in general, this lower concentration of ALP was less effective in restoring the allergy-protective effect. Since this concentration was still 5 times higher than present in raw cow's milk, these results might indicate that ALP is a promising candidate to be used as supplement to processed milk but is most likely not the component underlying the allergy-protective capacity of raw cow's milk.

Just like LF and ALP, addition of OPN to shop milk significantly lowered anaphylactic shock symptoms after intradermal challenge with OVA compared to shop milk alone. OPN-treated mice furthermore showed a reduction in both basal and OVA-specific basophil activation and thereby showed a similar basophil activation as raw milk-treated mice. OPN is one of the bioactive raw milk components recently gaining interest because of its promising effects when added to infant nutrition. Addition of bovine OPN to infant formula at concentrations similar to human milk resulted in less fever and a lower proinflammatory immune response than in infants fed regular formula (36). Effects on allergic diseases have not yet been explored, but the current preclinical study shows promising effects. The mechanism by which OPN affects allergic diseases remains to be elucidated. Besides a possible direct effect, OPN might also indirectly influence allergic outcomes since it has a high affinity for LF and can therefore act as a carrier protein that protects LF from proteolysis upon ingestion (37).

IgG was not able to affect the acute allergic skin response but did significantly reduce anaphylactic shock symptoms. Several studies have already demonstrated that preparations rich in IgG (e.g. bovine colostrum) can reduce infections in infants (38-40), but comparable studies on allergies are limited. As bioactive raw milk component, IgG is hypothesized to contribute to the observed protection against allergic diseases via the formation of immune

complexes (12). In a murine model, maternal transfer of IgG-allergen immune complexes in breast milk has been demonstrated to prevent OVA-induced allergic asthma in the offspring (41). Since bovine milk IgG was shown to bind to human allergens (42), these immune complexes might also be formed upon concurrent ingestion of bovine milk and allergenic proteins. Whether immune complex-dependent tolerance induction underlies the effects observed in the present study should be clarified in future research.

Shop milk supplemented with the combination of ALP, LF, OPN and IgG most closely resembled the allergy-protective raw milk effect. The acute allergic skin response and anaphylactic shock symptoms were reduced compared to shop milk alone and a similar effect was observed for basophil activation and OVA-specific IgE and IgG1 levels. Even though effects on acute allergic symptoms were comparable to shop milk supplemented with LF, the combination of the four bioactive whey proteins also reduced basophil activation and the humoral immune response. Together, ALP, LF, OPN and IgG therefore proved to be most effective in restoring the allergy-protective effect lost upon milk processing.

Because of the preliminary nature of this study, the bioactive whey proteins were added to shop milk in higher concentrations than present in raw milk (5× higher for ALP, LF, and OPN and 2× higher for IgG). The current study could therefore not conclude that these components are underlying the allergy-protective effects of raw cow's milk but does demonstrate that spiking heat-treated milk with bioactive whey proteins has great potential as alternative to raw cow's milk consumption. Despite these promising results, we have to be careful with their interpretation due to the lack of a strong allergic response in the positive control group. In contrast to many of our previous studies (10, 11, 18), and despite the widely used sensitization protocol, intradermal challenge with OVA did not increase acute allergic symptoms in OVA-sensitized allergic mice compared to PBS-sensitized control mice. The obtained results should therefore be confirmed in future studies. In addition, these studies should focus on investigating the allergy-protective potential of ALP, LF, OPN, and IgG at lower concentrations (i.e. the concentration present in raw cow's milk) to achieve a better understanding of the components responsible for the protective raw milk effect.

In conclusion, this study provides preliminary evidence that spiking heat-treated milk with bioactive whey proteins is a promising alternative to raw cow's milk consumption. The combination of the four bioactive whey proteins showed the greatest capacity to restore the allergy-protective effect lost upon milk processing since it reduced acute allergic symptoms, basophil activation, and OVA-IgE antibody levels. Of the four individual whey proteins tested, LF showed the strongest protective effects, although ALP, OPN, and IgG were also promising. Future studies should confirm whether fortification of industrially processed milk with bioactive whey proteins is indeed a promising preventive approach for allergic diseases.

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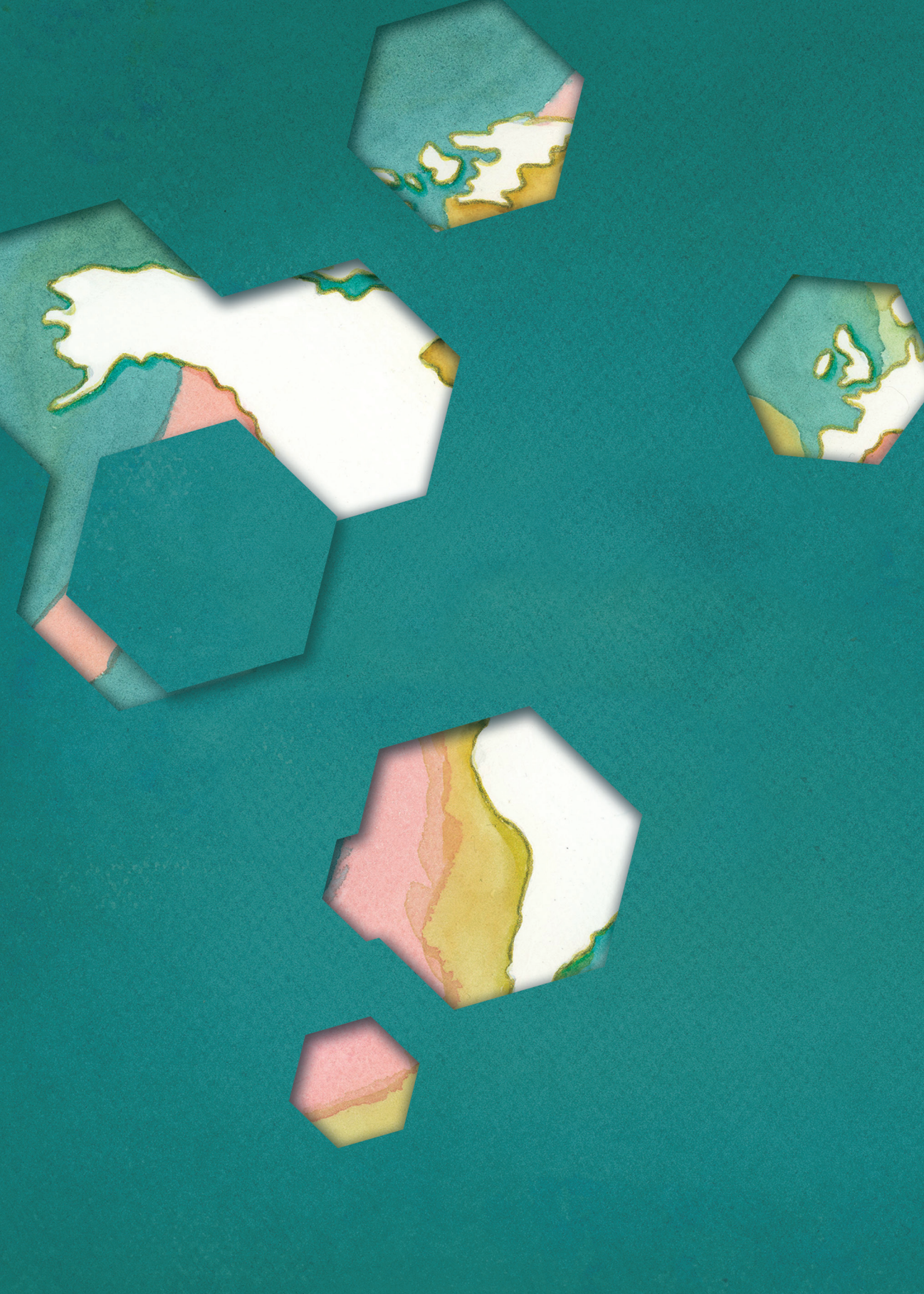
REFERENCES

1. Committee on Infectious Diseases & Committee on Nutrition & American Academy of Pediatrics. Consumption of raw or unpasteurized milk and milk products by pregnant women and children. *Pediatrics* (2014) 133(1): 175-9.
2. Braun-Fahrländer C and von Mutius E. Can farm milk consumption prevent allergic diseases? *Clin Exp Allergy* (2011) 41(1): 29-35.
3. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
4. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, *et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
5. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
6. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
7. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
8. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-1706 e13.
9. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T, *et al*. Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model. *Front Immunol* (2017) 8: 1045.
10. Abbring S, Ryan JT, Diks MAP, Hols G, Garssen J, and van Esch BCAM. Suppression of food allergic symptoms by raw cow's milk in mice is retained after skimming but abolished after heating the milk - A promising contribution of alkaline phosphatase. *Nutrients* (2019) 11(7): 1499.
11. Abbring S, Wolf J, Ayechu Muruzabal V, Diks MAP, Alashkar Alhamwe B, Alhamdan F, *et al*. Raw cow's milk reduces allergic symptoms in a murine model for food allergy - A potential role for epigenetic modifications. *Nutrients* (2019) 11(8): 1721.
12. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
13. Abbring S, Hols G, Garssen J, and van Esch BCAM. Raw cow's milk consumption and allergic diseases - The potential role of bioactive whey proteins. *Eur J Pharmacol* (2019) 843: 55-65.
14. Abbring S, Kusche D, Roos TC, Diks MAP, Hols G, Garssen J, *et al*. 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(7): 1013-25.
15. Bundesministerium der Justiz und für Verbraucherschutz. Verordnung über die Güteprüfung und Bezahlung der Anlieferungsmilch (Milch-Güteverordnung). (1980) Available from: http://www.gesetze-im-internet.de/milchg_v/index.html Accessed on: January 9 2019
16. Li XM, Schofield BH, Huang CK, Kleiner GI, and Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol* (1999) 103(2 Pt 1): 206-14.

17. Torrero MN, Larson D, Hubner MP, and Mitre E. CD200R surface expression as a marker of murine basophil activation. *Clin Exp Allergy* (2009) 39(3): 361-9.
18. van Esch BCAM, Abbring S, Diks MAP, Dingjan GM, Harthoorn LF, Vos AP, *et al*. Post-sensitization administration of non-digestible oligosaccharides and Bifidobacterium breve M-16V reduces allergic symptoms in mice. *Immun Inflamm Dis* (2016) 4(2): 155-65.
19. Chatterton DE, Nguyen DN, Bering SB, and Sangild PT. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int J Biochem Cell Biol* (2013) 45(8): 1730-47.
20. Legrand D. Lactoferrin, a key molecule in immune and inflammatory processes. *Biochem Cell Biol* (2012) 90(3): 252-68.
21. Giansanti F, Panella G, Leboffe L, and Antonini G. Lactoferrin from milk: nutraceutical and pharmacological properties. *Pharmaceuticals* (2016) 9(4): 61.
22. Sherman MP, Bennett SH, Hwang FF, and Yu C. Neonatal small bowel epithelia: enhancing anti-bacterial defense with lactoferrin and Lactobacillus GG. *Biometals* (2004) 17(3): 285-9.
23. Oda H, Wakabayashi H, Yamauchi K, and Abe F. Lactoferrin and bifidobacteria. *Biometals* (2014) 27(5): 915-22.
24. Bjorksten B, Sepp E, Julge K, Voor T, and Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* (2001) 108(4): 516-20.
25. Sjogren YM, Jenmalm MC, Bottcher MF, Bjorksten B, and Sverremark-Ekstrom E. Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clin Exp Allergy* (2009) 39(4): 518-26.
26. Liao Y, Jiang R, and Lonnerdal B. Biochemical and molecular impacts of lactoferrin on small intestinal growth and development during early life. *Biochem Cell Biol* (2012) 90(3): 476-84.
27. Lonnerdal B, Jiang R, and Du X. Bovine lactoferrin can be taken up by the human intestinal lactoferrin receptor and exert bioactivities. *J Pediatr Gastroenterol Nutr* (2011) 53(6): 606-14.
28. Takakura N, Wakabayashi H, Yamauchi K, and Takase M. Influences of orally administered lactoferrin on IFN-gamma and IL-10 production by intestinal intraepithelial lymphocytes and mesenteric lymph-node cells. *Biochem Cell Biol* (2006) 84(3): 363-8.
29. Faria AM and Weiner HL. Oral tolerance. *Immunol Rev* (2005) 206: 232-59.
30. Hawrylowicz CM and O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* (2005) 5(4): 271-83.
31. Rankin SA, Christiansen A, Lee W, Banavara DS, and Lopez-Hernandez A. Invited review: the application of alkaline phosphatase assays for the validation of milk product pasteurization. *J Dairy Sci* (2010) 93(12): 5538-51.
32. Lalles JP. Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr Rev* (2014) 72(2): 82-94.
33. Beumer C, Wulferink M, Raaben W, Fiechter D, Brands R, and Seinen W. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. *J Pharmacol Exp Ther* (2003) 307(2): 737-44.
34. Whitehouse JS, Riggle KM, Purpi DP, Mayer AN, Pritchard KA, Jr., Oldham KT, *et al*. The protective role of intestinal alkaline phosphatase in necrotizing enterocolitis. *J Surg Res* (2010) 163(1): 79-85.
35. Tuin A, Poelstra K, de Jager-Krikken A, Bok L, Raaben W, Velders MP, *et al*. Role of alkaline phosphatase in colitis in man and rats. *Gut* (2009) 58(3): 379-87.
36. Lonnerdal B, Kvistgaard AS, Peerson JM, Donovan SM, and Peng YM. Growth, nutrition, and cytokine response of breast-fed infants and infants fed formula with added bovine osteopontin. *J Pediatr Gastroenterol Nutr*

(2016) 62(4): 650-7.

37. Yamniuk AP, Burling H, and Vogel HJ. Thermodynamic characterization of the interactions between the immunoregulatory proteins osteopontin and lactoferrin. *Mol Immunol* (2009) 46(11-12): 2395-402.
38. Timby N, Hernell O, Vaarala O, Melin M, Lonnerdal B, and Domellof M. Infections in infants fed formula supplemented with bovine milk fat globule membranes. *J Pediatr Gastroenterol Nutr* (2015) 60(3): 384-9.
39. Saad K, Abo-Elela MG, El-Baseer KA, Ahmed AE, Ahmad FA, Tawfeek MS, *et al*. Effects of bovine colostrum on recurrent respiratory tract infections and diarrhea in children. *Medicine* (2016) 95(37): e4560.
40. Ulfman LH, Leusen JHW, Savelkoul HFJ, Warner JO, and van Neerven RJJ. Effects of bovine immunoglobulins on immune function, allergy, and infection. *Front Nutr* (2018) 5: 52.
41. Mosconi E, Rekima A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, *et al*. Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. *Mucosal Immunol* (2010) 3(5): 461-74.
42. Collins AM, Roberton DM, Hosking CS, and Flannery GR. Bovine milk, including pasteurised milk, contains antibodies directed against allergens of clinical importance to man. *Int Arch Allergy Appl Immunol* (1991) 96(4): 362-7.



CHAPTER 8

Milk processing increases the allergenicity of cow's milk – Preclinical evidence supported by a human proof-of-concept provocation pilot

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ABSTRACT

Several studies demonstrated the adverse effect of milk processing on the allergy-protective capacity of raw cow's milk. Whether milk processing also affects the allergenicity of raw milk is hardly investigated. In the present study, we assessed the allergenicity of raw (unprocessed) and processed cow's milk in a murine model for food allergy as well as in cow's milk allergic children. C3H/HeOuJ mice were either sensitized to whole milk (raw cow's milk, heated raw cow's milk, or shop milk [store-bought milk]) and challenged with cow's milk protein or they were sensitized and challenged with whey proteins (native or heated). Acute allergic symptoms, mast cell degranulation, allergen-specific IgE levels, and cytokine concentrations were determined upon challenge. Cow's milk allergic children were tested in an oral provocation pilot with organic raw and conventional shop milk. Mice sensitized to raw milk showed fewer acute allergic symptoms upon intradermal challenge than mice sensitized to processed milk. The acute allergic skin response was low (103 ± 8.5 μm vs 195 ± 17.7 μm for heated raw milk, $P < 0.0001$ and vs 149 ± 13.6 μm for shop milk, $P = 0.0316$), and there were no anaphylactic shock symptoms and no anaphylactic shock-induced drop in body temperature. Moreover, allergen-specific IgE levels and Th2 cytokines were significantly lower in raw milk-sensitized mice. Interestingly, the reduced sensitizing capacity was preserved in the isolated native whey protein fraction of raw milk. Besides, native whey protein challenge diminished allergic symptoms in mice sensitized to heated whey proteins. In an oral provocation pilot, cow's milk allergic children tolerated raw milk up to 50 mL whereas they only tolerated 8.6 ± 5.3 mL shop milk ($P = 0.0078$). This study demonstrates that raw (unprocessed) cow's milk and native whey proteins have a lower allergenicity than their processed counterparts. The preclinical evidence in combination with the human proof-of-concept provocation pilot provides evidence that milk processing negatively influences the allergenicity of milk.

INTRODUCTION

Thousands of years ago, humans started to consume cow's milk as part of their nutrition. At that time, cow's milk was consumed raw, but since the late 19th century it has been pasteurized and homogenized (1). This industrial milk processing extends shelf life and, more importantly, reduces the risk of milk-borne infections caused by pathogenic bacteria like *Mycobacterium tuberculosis*, *Listeria*, *Salmonella*, *Campylobacter*, Enterohemorrhagic *Escherichia coli* (EHEC), and Shigatoxigenic *Escherichia coli* (STEC) (2). However, milk processing can also have disadvantages. Pasteurization, for instance, structurally alters heat-sensitive milk components, like proteins, which might subsequently lose functionality (3). In addition, homogenization changes the milk fat structure and thereby it might alter allergen presentation to the immune system (4, 5). So even though milk processing ensures microbial safety, it also affects functional milk proteins which might consequently lose their beneficial health properties.

In Germany, a long tradition of the consumption of raw, unprocessed, farm milk is existing. A survey on biodynamic dairy farms reported that (part of the) consumers bought organic raw milk because of a better tolerance and beneficial health effects (6). Beneficial health effects of raw cow's milk consumption are mainly described for asthma and allergic diseases. Raw cow's milk consumption has been associated with a reduced risk of these diseases (7-11). The body of evidence for this protective effect is accumulating with epidemiological as well as preclinical evidence (12, 13). Interestingly, the asthma- and allergy-protective effect of raw cow's milk seems to be abolished by milk processing. Heat treatment, in particular, appears to impact the protective effect, suggesting the importance of heat-sensitive milk components, such as whey proteins (7, 12, 13). From a variety of these whey proteins, it is believed that they might contribute to the protective effects of raw cow's milk (14, 15). Whether these components by themselves have the capacity to reduce the asthma and allergy risk remains to be elucidated.

The adverse effect of milk processing on the asthma- and allergy-protective capacity of raw cow's milk is, as mentioned before, demonstrated by several studies (7, 12, 13). Whether milk processing also affects allergen presentation to the immune system and thus influences the allergenic potential of the milk is hardly investigated. Heating of the whey proteins α-lactalbumin and β-lactoglobulin induces the formation of aggregates which seem to promote allergic sensitization by shifting uptake from enterocytes to Peyer's patches (16). In addition, homogenization might increase the allergenicity of the milk due to disintegration of casein micelles and milk fat globules (5). However, compelling evidence showing that milk processing affects the allergenicity of milk is still lacking.

In addition to the existing epidemiological evidence showing a tolerogenic feature of raw cow's milk, the present study investigated whether the allergenicity of raw (unprocessed) and processed cow's milk differs in a murine model for food allergy. Since several studies have speculated about the whey fraction of raw cow's milk containing potential allergy-protective components, we also assessed the allergenicity of native and heated whey proteins. In addition, we performed a proof-of-concept provocation pilot using a similar, organic, raw cow's milk in cow's milk allergic children.

MATERIALS & METHODS

Mice

Four-week-old, specific pathogen-free, female C3H/HeOuJ mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Upon arrival, mice were randomly assigned to the control or experimental groups. They were housed at the animal facility of Utrecht University (Utrecht, The Netherlands) in filter-topped makrolon cages (n = 6-8/cage) on a 12 h light/dark cycle with access to food and water *ad libitum*. All animal procedures were approved by the Ethical Committee for Animal Research of the Utrecht University and conducted in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes (DEC 2014.II.12.107 & AVD108002015346).

Milk and whey proteins

Raw milk used was an organic raw cow's milk with a fat content between 3.8% and 4.2% (due to seasonal variation) collected from a biodynamic farm legally allowed to sell raw milk (organic '*Vorzugsmilch*' (17); Hof Dannwisch, Horst, Germany). After collection, part of the raw milk was heated for 10 min at 80 °C in a water bath to obtain the heated raw cow's milk used. From heating at 80 °C, it is known that it will result in structural changes in proteins with immunomodulatory capacities which might subsequently lose functionality, but it will also denature β-lactoglobulin and α-lactalbumin. The shop milk (store-bought milk) used was a conventional pasteurized and homogenized milk standardized at 3.8% fat (EDEKA, Germany). All milk types had a protein level of around 3.5 g/100 mL, with no difference between raw and shop milk (as determined by using the Pierce BCA Protein Assay Kit standardized to bovine serum albumin [BSA] according to the manufacturer's protocol [Thermo Fisher Scientific, Paisley, Scotland]). Processed cow's milk protein (CMP) was obtained from DMV International (Veghel, The Netherlands). These are solely caseins and whey proteins in an 80:20 ratio. Native whey proteins (nWP; 3% denaturation) were isolated from raw cow's milk. Heated whey proteins (hWP; 73% denaturation; produced for experimental purposes only; Danone Nutricia Research, Utrecht, The Netherlands) were obtained by heating nWP for 60 s at 100 °C.

Experimental design – Oral sensitization of mice to raw and processed cow's milk

A schematic overview of the experimental design is depicted in **Figure 1A**. After one week habituation, mice (*n* = 8/group) were sensitized intragastrically (i.g.) by using a blunt needle with 0.5 mL raw cow's milk, heated raw cow's milk, or shop milk using 10 µg cholera toxin (CT; List Biological Laboratories, Campbell, CA, USA) as an adjuvant. Since sensitization to whole milk was never performed before in this model, a sensitized control group (*n* = 8), which received 17.5 mg processed CMP (equivalent to the amount of protein present in 0.5 mL cow's milk) dissolved in 0.5 mL PBS (17.5 mg CMP/0.5 mL PBS + 10 µg CT), was included (18). Sham-sensitized control mice (*n* = 6) received CT alone (10 µg/0.5 mL PBS). Mice were sensitized once a week for five consecutive weeks (on days 0, 7, 14, 21, and 28). Five days after the last sensitization (day 33), all mice were challenged intradermally (i.d.) in the ear pinnae of both ears with 10 µg CMP in 20 µL PBS to determine the acute allergic response. On the same day, mice were challenged i.g. with 50 mg CMP in 0.5 mL PBS. Sixteen hours after the oral challenge, blood samples were collected via cheek puncture. Mice were subsequently killed by cervical dislocation, and organs were collected for *ex vivo* analysis. The raw milk used to sensitize mice was obtained from the same farm that was selected five years earlier to deliver the milk for the human provocation pilot. The mouse study was partly based on results of the human study.

Experimental design – Oral sensitization and challenge of mice with native and heated whey proteins

In the second experiment, nWP and hWP were used to sensitize and challenge mice (**Figure 1B**). The experimental design is comparable to the one described above. Shortly, mice (*n* = 8) were sensitized i.g. once a week for five consecutive weeks to nWP or hWP (20 mg/0.5 mL PBS + 10 µg CT). Five days after the last sensitization (day 33), mice were challenged both i.d. and i.g. with nWP or hWP (10 µg/20 µL PBS and 50 mg/0.5 mL PBS, respectively) to assess the allergic response. Mice were killed by cervical dislocation 16 h after the i.g. challenge.

Evaluation of the allergic response

To determine the acute allergic skin response, mice were i.d. challenged with the allergen (10 µg allergen/20 µL PBS) in the ear pinnae of both ears. Ear thickness (in duplicate for each ear) was measured before and 1 h after the i.d. challenge using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). Ear swelling, expressed as Δ µm, was subsequently calculated by subtracting the mean basal ear thickness before i.d. challenge from the mean ear thickness measured 1 h after the i.d. challenge. The i.d. challenge and the ear measurements were performed in anesthetized mice (using inhalation of isoflurane; Abbott, Breda, The Netherlands). Severity of anaphylactic shock symptoms was scored 30 min after the i.d. challenge by using a validated scoring table (19). The anaphylactic shock-induced drop in body temperature was also measured 30 min after i.d. challenge using a rectal thermometer. All measurements were performed blinded.

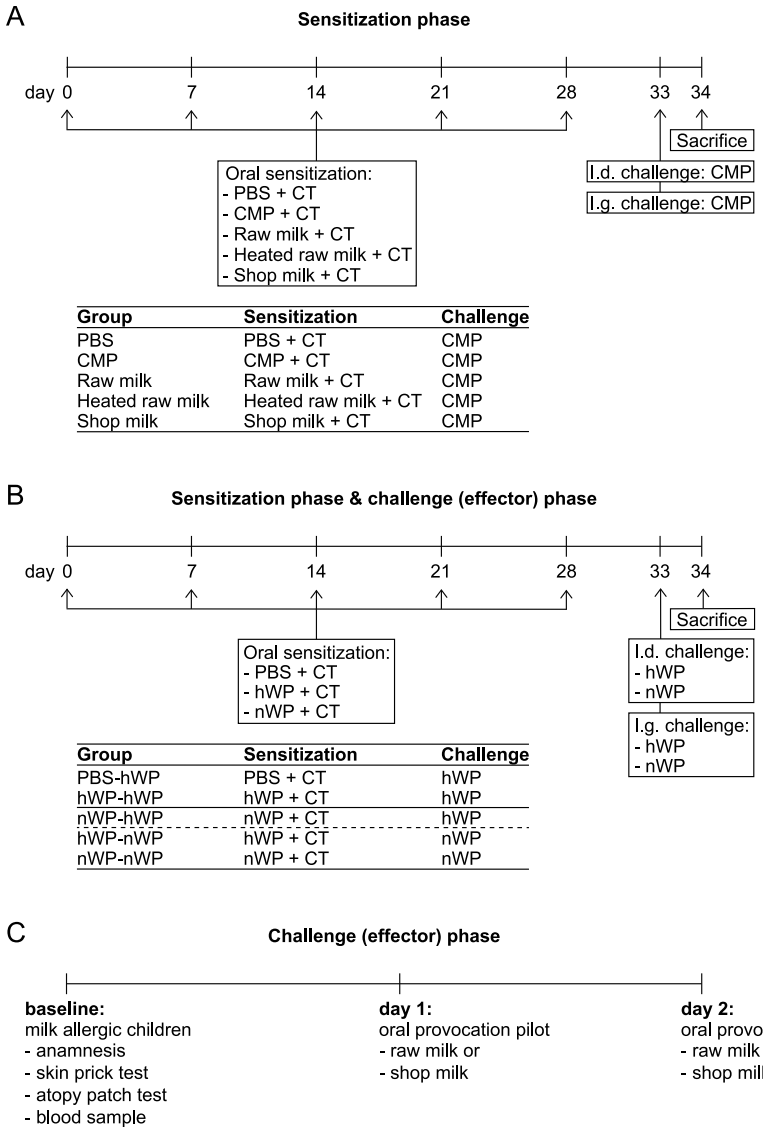

Figure 1. Schematic overview of the *in vivo* experiments and the proof-of-concept provocation pilot. (**A**) Experimental design oral sensitization of mice to raw and processed cow's milk. (**B**) Experimental design oral sensitization and challenge of mice with native and heated whey proteins. To distinguish between effects on the sensitization and challenge phase, groups were split, and data were analyzed separately. The first two groups, indicated above the solid line, are control groups included in all analyses. The third group, indicated above the dashed line, is only included in analyses on the sensitization phase. The last two groups are only included in analyses on the challenge phase. (**C**) Protocol proof-of-concept provocation pilot with organic raw cow's milk and conventional shop milk in cow's milk allergic children. CT, cholera toxin; CMP, cow's milk protein; i.d., intradermal; i.g., intragastric; hWP, heated whey proteins; nWP, native whey proteins.

Measurements of serum allergen-specific IgE and mMCP-1

Blood was collected via cheek puncture 16 h after oral challenge and centrifuged at 10,000 *g* for 10 min. Serum was obtained and stored at -20 °C until analysis of allergen-specific IgE and mucosal mast cell protease-1 (mMCP-1) levels by means of ELISA. Determination of CMP- (i.e. caseins and whey proteins), hWP-, nWP-, and raw cow's milk-specific IgE antibodies was performed as previously described (20), with few alterations. Briefly, high binding Costar 9018 plates (Corning Inc., New York, NY, USA) were coated with 20 µg/mL caseins or whey proteins in carbonate/bicarbonate coating buffer (0.05 M, pH 9.6; Sigma-Aldrich, Zwijndrecht, The Netherlands) and incubated overnight at 4 °C. For the determination of raw cow's milk-specific IgE antibodies, plates were coated with raw cow's milk (diluted 1750x to obtain a protein concentration of 20 µg/mL). After overnight incubation, plates were washed and blocked for 1 h with PBS/1% BSA (Sigma-Aldrich). Serum samples were subsequently incubated for 2 h. After washing, plates were incubated with biotinylated rat anti-mouse IgE detection antibody (1 µg/mL; BD Biosciences, Alphen aan de Rijn, The Netherlands) for 1.5 h. Plates were then washed, incubated for 45 min with streptavidin-horseradish peroxidase (0.5 µg/mL; Sanquin, Amsterdam, The Netherlands), washed again and developed using *o*-phenylenediamine (Sigma-Aldrich). The reaction was stopped by 4 M H_2SO_4, and absorbance was measured at 490 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands). Concentrations of mMCP-1 were measured using a mMCP-1 Ready-SET-Go!® ELISA (eBioscience, Breda, The Netherlands) according to the manufacturer's instructions.

***Ex vivo* antigen-specific stimulation of splenocytes to determine cytokine profiles**

Single cell splenocyte suspensions were obtained by passing spleen samples through a 70 µm nylon cell strainer using a syringe. The splenocyte suspension was rinsed with RPMI 1640 medium (Lonza, Verviers, Belgium) and incubated with lysis buffer (8.3 g NH_4Cl, 1 g KHC_3O, and 37.2 mg EDTA dissolved in 1 L demi water, filter sterilized) to remove red blood cells. The reaction was stopped by adding RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands), penicillin (100 U/mL)/streptomycin (100 µg/mL; Sigma-Aldrich), and β-mercaptoethanol (20 µM; Thermo Fisher Scientific). Splenocytes were subsequently resuspended in this culture medium. For the *ex vivo* antigen-specific restimulation assay, splenocytes (8×10^5 cells/well) were cultured in culture medium with or without 500 µg/mL CMP/hWP/nWP. Supernatant was harvested after four days of culture (37 °C, 5% CO_2), and stored at -20 °C until cytokine analysis. Measurements of IL-5, IL-13, IL-10, and IFNγ were performed by means of ELISA according to the protocol described above for IgE. Purified rat anti-mouse antibodies (1 µg/mL for IL-5 and IFNγ, and 2 µg/mL for IL-13 and IL-10), recombinant mouse cytokines, and biotinylated rat anti-mouse antibodies (1 µg/mL for IL-5, IL-10, and IFNγ, and 400 ng/mL for IL-13) were purchased at BD Biosciences.

Experimental design – Proof-of-concept human provocation pilot with organic raw cow's milk in cow's milk allergic children

This study was part of a larger research project of the University of Kassel (Germany) focusing on the difference between organic and conventional milk quality, including effects of production and processing of organic (raw) milk on human health. In the current study, the largest contrast in milk quality (organic raw milk vs conventional shop milk) was used to determine tolerance in cow's milk allergic children. The study was reviewed and approved by the ethical committee of the 'Ärztekammer' Niedersachsen (Hannover, Germany; Bo/06/2009). In total, 11 children with parent-reported cow's milk allergy were recruited from the Reha Klinik, Interdisciplinary Centre for Dermatology, Pneumology, and Allergology in Neuharlingersiel (Germany) and informed consent was obtained before enrollment. To confirm milk allergy diagnosis, a skin prick test (expressed as wheal diameter in mm; measured after 20 min) and an atopy patch test (APT; expressed as negative [-] or positive [+, ++, +++, ++++] reaction, qualified according to the APT reading criteria of the European Task Force on Atopic Dermatitis [ETFAD] (21); measured after 48 h) were performed with a commercial diagnostic milk-prick-solution (ALK-Abelló Arzneimittel GmbH, Hamburg, Germany). In addition, a blood sample was taken to determine total and cow's milk-specific serum IgE levels. To determine differences in milk tolerance level, each child was subsequently tested in a double-blind placebo-controlled oral provocation pilot with raw milk as well as shop milk. All children were tested within a period of one year. The day before testing, fresh raw milk was delivered from the biodynamic farm and shop milk was bought from the local shop. Each child was tested for each milk type in random order on two consecutive days. Since the study was performed double-blind, milk was offered by a nurse in increasing quantities every 30 min. The medical doctor judged the allergic symptoms and gave permission for the next dose at the end of the time interval. Based on this judgement, milk consumption was increased to a maximum of 50 mL.

Statistical analysis

Data are presented as mean ± SEM or as individual data points when data were not normally distributed. In the first *in vivo* experiment (oral sensitization of mice to raw and processed cow's milk; **Figure 1A**), differences between pre-selected groups were statistically analyzed using one- or two-way ANOVA, followed by Bonferroni's multiple comparisons test. For mMCP-1 concentrations, log-transformed data were used to obtain normality for one-way ANOVA. Anaphylactic shock scores and serum IgE levels were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons test for pre-selected groups since data did not obtain normality. For the second *in vivo* experiment (oral sensitization and challenge of mice with native and heated whey proteins; **Figure 1B**), groups were split and data were analyzed separately to discriminate between effects on the sensitization and challenge (effector) phase. Differences compared to the hWP-hWP group were statistically analyzed using one- or two-way ANOVA, followed by Dunnett's multiple comparisons test. Serum IgE levels were analyzed

using Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test. Differences in milk tolerance level in the proof-of-concept provocation pilot (**Figure 1C**) were determined using a Wilcoxon signed-rank test. Results were considered statistically significant when $P < 0.05$. Analyses were performed using GraphPad Prism software (version 7; GraphPad Software, San Diego, CA, USA).

RESULTS

Mice sensitized to raw milk show fewer allergic symptoms upon CMP challenge

As expected, mice sensitized to processed cow's milk protein (CMP; sensitized control mice) showed increased allergic symptoms upon i.d. challenge with CMP compared to mice sensitized to PBS (sham-sensitized control mice). This increase in allergic symptoms was characterized by an increased acute allergic skin response, increased anaphylactic shock symptoms, and an anaphylactic shock-induced drop in body temperature (**Figures 2A-C**). To determine whether milk processing affects the capacity of milk to induce allergic symptoms to CMP, mice were sensitized to raw cow's milk, heated raw cow's milk, or shop milk. Mice sensitized to raw milk showed little allergic symptoms upon i.d. challenge with CMP; the acute allergic skin response was low, there were no anaphylactic shock symptoms, and the body temperature remained high (**Figures 2A-C**). Sensitization to the processed milk types, on the contrary, increased acute allergic skin response and anaphylactic shock symptoms and caused an anaphylactic shock-induced drop in body temperature (**Figures 2A-C**).

Lower allergen-specific IgE levels in raw milk-sensitized mice

To assess whether the reduced allergic symptoms in raw milk-sensitized mice coincided with reduced allergic sensitization, serum allergen-specific IgE levels were measured. Since caseins and whey proteins are the main milk allergens, specific IgE levels against these proteins were determined. Both casein- and whey-specific IgE levels were low in raw milk-sensitized mice and increased significantly when processed milk was used to sensitize mice (**Figures 2D,E**). Since the caseins and whey proteins used to determine these IgE levels were derived from a heated source, one could argue that conformational changes induced by heating limit the detection of IgE antibodies formed to raw milk. Therefore, raw milk-specific IgE levels were also measured. However, as shown in **Figure 2F**, raw milk-specific IgE levels were also low in the raw milk group. In addition, serum mMCP-1 concentration was measured to assess mucosal mast cell degranulation. Increased mMCP-1 concentrations were observed in sensitized control mice compared to sham-sensitized control mice (**Figure 2G**). mMCP-1 did, however, not differ between milk groups (**Figure 2G**).

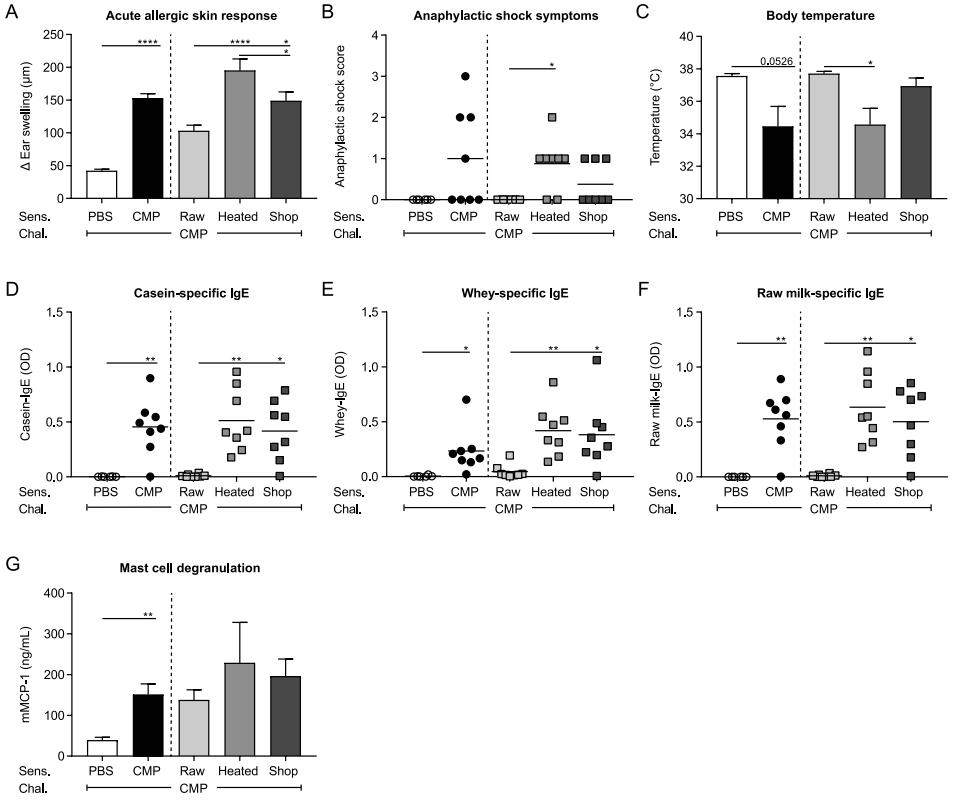

Figure 2. Fewer allergic symptoms and lower IgE levels after sensitization to raw milk. (**A**) The acute allergic skin response (Δ ear swelling) measured 1 h after i.d. challenge. (**B**) Anaphylactic shock scores and (**C**) body temperature determined 30 min after i.d. challenge. (**D**) Serum casein- (**E**) whey-, and (**F**) raw milk-specific IgE antibody levels and (**G**) serum mMCP-1 concentrations measured 16 h after i.g. challenge. Data are presented as mean ± SEM or as individual data points when data were not normally distributed, $n = 6$ in PBS group and $n = 8$ in all other groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, as analyzed with one-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups (**A, C, G**) or Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test for pre-selected groups (**B, D-F**). Sens., sensitization; chal., challenge; CMP, cow's milk protein; raw, raw cow's milk; heated, heated raw cow's milk; shop, shop milk; OD, optical density; mMCP-1; mucosal mast cell protease-1; i.d., intradermal; i.g., intragastric.

Raw milk sensitization inhibits Th2-related cytokine production after *ex vivo* stimulation of splenocytes with CMP

To investigate whether sensitization to different milk types affected T cell functionality, splenocytes were stimulated *ex vivo* with CMP and allergen-induced cytokine concentrations were measured. Low cytokine levels were observed in sham-sensitized control mice, whereas sensitized control mice showed a, CMP-specific, increase in IL-5, IL-13, IL-10, and IFNγ (**Figures 3A-D**). Secretion of Th2-related cytokines IL-5 and IL-13 markedly increased upon

CMP stimulation in mice sensitized to processed milk, whereas secretion remained low in mice sensitized to raw milk (**Figures 3A,B**). A similar pattern was observed for IL-10 (**Figure 3C**). IFNγ production was not affected by the different milk types (**Figure 3D**).

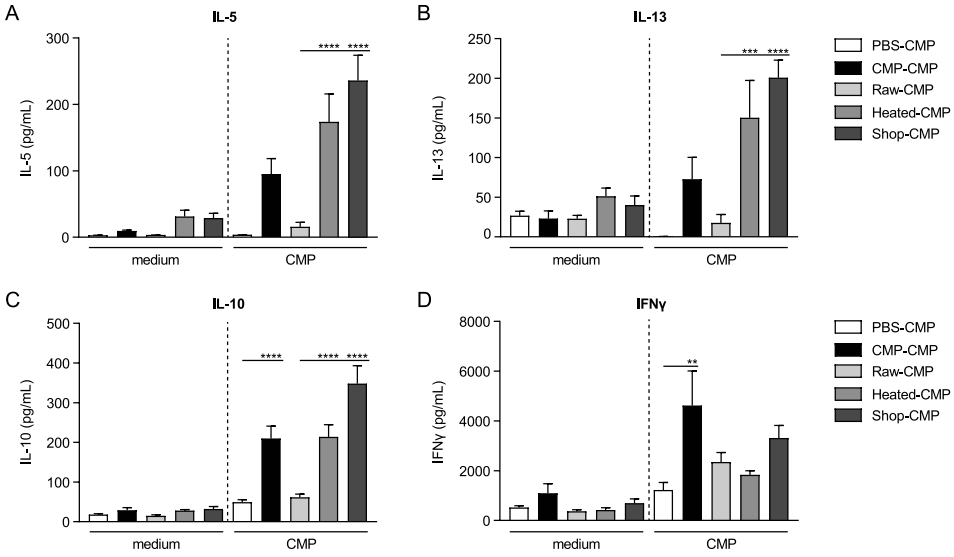

Figure 3. Th2-related cytokine production after *ex vivo* stimulation of splenocytes with CMP was inhibited in raw milk-sensitized mice. (**A**) IL-5 (sensitization: $P < 0.0001$, stimulation: $P < 0.0001$, interaction: $P < 0.0001$), (**B**) IL-13 (sensitization: $P < 0.0001$, stimulation: $P < 0.0001$, interaction: $P < 0.001$), (**C**) IL-10 (sensitization: $P < 0.01$, stimulation: $P < 0.0001$, interaction: $P < 0.0001$), and (**D**) IFNγ (sensitization: $P < 0.0001$, stimulation: $P < 0.0001$, interaction: ns) concentrations were measured in supernatant after *ex vivo* stimulation of splenocytes with medium or CMP for four days (37 °C, 5% CO_2). Data are presented as mean ± SEM, $n = 5$-6 in PBS group and $n = 7$-8 in all other groups. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, as analyzed with two-way ANOVA followed by Bonferroni's multiple comparisons test for pre-selected groups. CMP, cow's milk protein; raw, raw cow's milk; heated, heated raw cow's milk; shop, shop milk; ns, not significant.

To determine whether sensitization with the different milk types affected the microbiota composition, metabolic activity of the microbiome was assessed by measuring short-chain fatty acid concentrations in caecum. However, no differences were observed between groups in acetic acid, propionic acid, and butyric acid concentrations (**Figures S1A-C**).

Nativity of whey proteins important to reduce allergic sensitization

Previous studies have speculated the whey protein fraction of raw milk may be a source of the allergy-protective components (7, 12, 15). To assess whether the reduced sensitizing capacity of raw milk is still present when only looking at the whey protein fraction of the milk, a similar experiment was conducted with native and heated whey proteins (**Figure 1B**). **Figure 4A**

demonstrates that the acute allergic skin response was indeed reduced in mice sensitized to nWP compared to mice sensitized to hWP when challenged with hWP. This reduction coincided with reduced mucosal mast cell degranulation (**Figure 4B**). Whey protein-specific IgE antibody levels were increased in hWP-sensitized mice, whereas no significant levels were observed in nWP-sensitized mice (**Figures 4C,D**). This effect was observed for both hWP- and nWP-specific IgE (**Figures 4C,D**), suggesting that heating did not induce conformational changes in these proteins affecting B cell epitopes and hence IgE binding. Allergen-induced IL-5, IL-13, and IL-10 production was also significantly lower in the nWP group compared to the hWP group (**Figures 4E-G**). Allergen-induced IFNγ production did not differ between groups (**Figure 4H**).

Native whey proteins diminish allergic symptoms in heated whey protein-sensitized mice

The data presented (**Figure 4**) suggest nWP indeed have a lower sensitizing capacity than hWP (comparable to raw milk; **Figures 2 and 3**). To determine whether nWP also have a lower capacity to induce an allergic response when sensitization to hWP already occurred, hWP-sensitized mice were challenged with nWP. Challenge with nWP induced a lower acute allergic skin response than challenge with hWP, in hWP-sensitized mice (**Figure 5A**). It did not reduce mast cell degranulation and whey protein-specific IgE levels but it did inhibit IL-5, IL-13, and IL-10 production by splenocytes after allergen-specific stimulation (**Figures 5B-G**). Allergen-specific stimulation did not induce differences between groups in IFNγ secretion (**Figure 5H**). To investigate whether allergic symptoms were induced in mice exclusively exposed to nWP, a group sensitized and challenged with nWP was included. However, this group showed similar protective effects (**Figures 5A-H**).

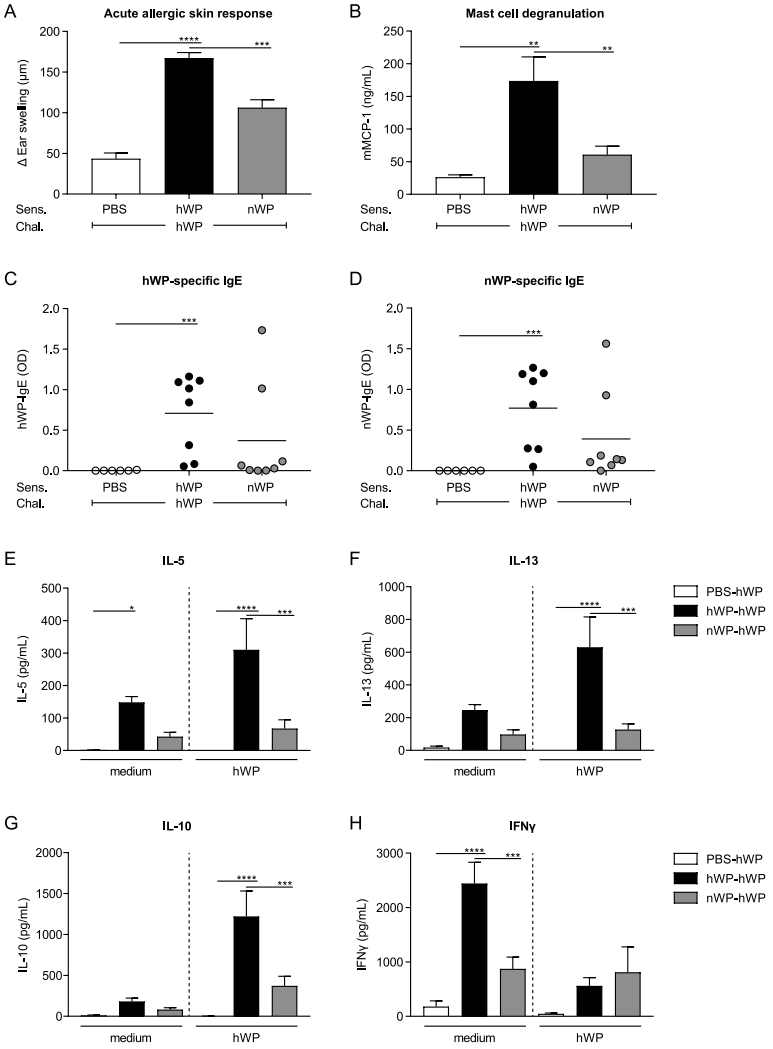

Figure 4. Reduced allergic response after sensitization to native whey proteins. (**A**) The acute allergic skin response (Δ ear swelling) measured 1 h after i.d. challenge. (**B**) Serum mMCP-1 concentrations and (**C**) hWP- and (**D**) nWP-specific IgE antibody levels measured 16 h after i.g. challenge. (**E**) IL-5 (sensitization: $P < 0.0001$, stimulation: ns, interaction: ns), (**F**) IL-13 (sensitization: $P < 0.0001$, stimulation: ns, interaction: $P < 0.05$), (**G**) IL-10 (sensitization: $P < 0.0001$, stimulation: $P < 0.001$, interaction: $P < 0.01$), and (**H**) IFNγ (sensitization: $P < 0.001$, stimulation: $P < 0.01$, interaction: $P < 0.01$) concentrations measured in supernatant after *ex vivo* stimulation of splenocytes with medium or hWP for four days (37 °C, 5% CO_2). Data are presented as mean ± SEM, n = 5-6 in PBS group and n = 7-8 in all other groups. *$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, compared to the hWP-hWP group as analyzed with one-way ANOVA followed by Dunnett's multiple comparisons test (**A, B**), Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test (**C, D**), or two-way ANOVA followed by Dunnett's multiple comparisons test (**E-H**). Sens., sensitization; chal., challenge; hWP, heated whey proteins; nWP, native whey proteins; mMCP-1; mucosal mast cell protease-1; OD, optical density; i.d., intradermal; i.g., intragastric; ns, not significant.

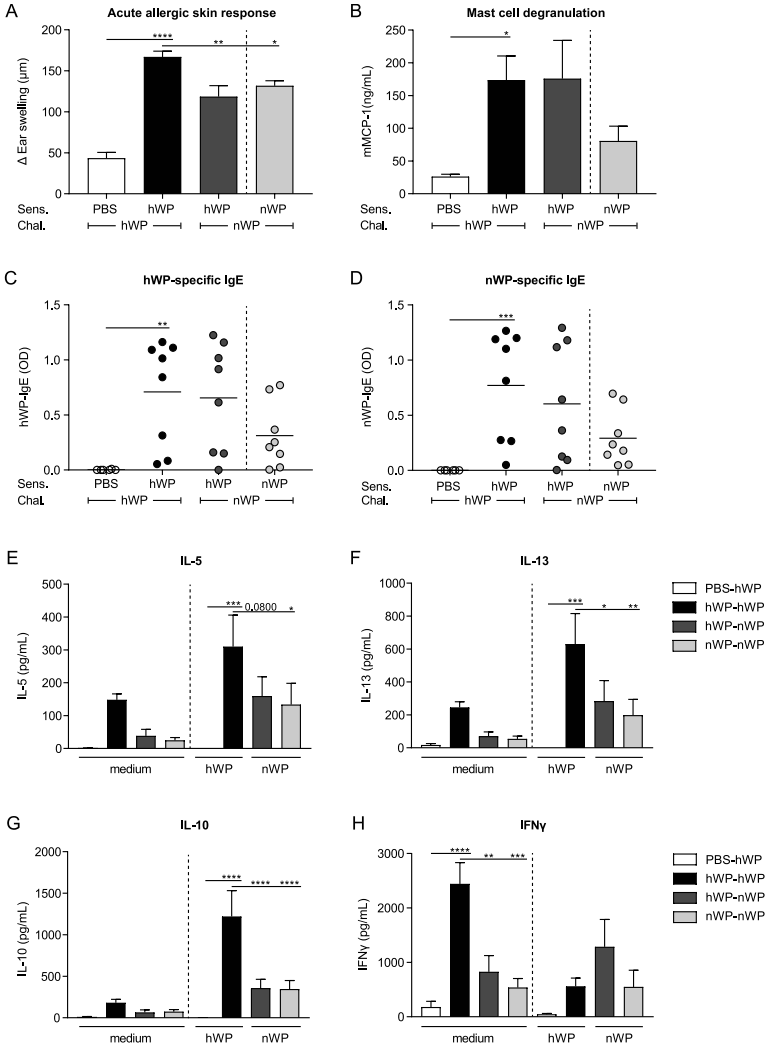

Figure 5. Challenge with native whey proteins diminished the allergic response in heated whey protein-sensitized mice. (**A**) The acute allergic skin response measured as Δ ear swelling 1 h after i.d. challenge. (**B**) Serum mMCP-1 concentrations 16 h after i.g. challenge. (**C**) hWP- and (**D**) nWP-specific IgE levels measured 16 h after i.g. challenge. (**E**) IL-5 (sensitization: $P < 0.001$, stimulation: $P < 0.01$, interaction: ns), (**F**) IL-13 (sensitization: $P < 0.001$, stimulation: $P < 0.01$, interaction: ns), (**G**) IL-10 (sensitization: $P < 0.0001$, stimulation: $P < 0.0001$, interaction: $P < 0.01$), and (**H**) IFNγ (sensitization: $P < 0.001$, stimulation: ns, interaction: $P < 0.01$) concentrations measured in supernatant after *ex vivo* stimulation of splenocytes with medium, hWP, or nWP for four days (37 °C, 5% CO_2). Data are presented as mean ± SEM, n = 5-6 in PBS group and n = 7-8 in all other groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, compared to the hWP-hWP group as analyzed with one-way ANOVA followed by Dunnett's multiple comparisons test (**A, B**), Kruskal-Wallis test for non-parametric data followed by Dunn's multiple comparisons test (**C, D**), or two-way ANOVA followed by Dunnett's multiple comparisons test (**E-H**). Sens., sensitization; chal., challenge; hWP, heated whey proteins; nWP, native whey proteins; mMCP-1; mucosal mast cell protease-1; OD, optical density; i.d., intradermal; i.g., intragastric; ns, not significant.

Reduced allergenic potential of organic raw milk in cow's milk allergic children

In addition, children diagnosed with cow's milk allergy were tested in their reaction to organic raw cow's milk and conventional shop milk. Eleven children were included in this proof-of-concept double-blind placebo-controlled provocation pilot (results presented in **Table 1**). Two children (patients 6 and 7) were excluded from analysis since their milk allergy could not be confirmed in the skin prick test (0 mm). The remaining nine children (1.5 ± 0.3 [mean ± SEM] years of age) developed a wheal diameter of 6.7 ± 1.0 mm in the skin prick test and showed an average patch test result between ++ and +++ (2.4 ± 0.2). From the six children of which IgE levels were measured, the total serum IgE level was 115.4 ± 43.4 kU/L and the cow's milk-specific serum IgE level was 10.4 ± 3.4 kU/L. In the oral provocation pilot, all children could tolerate the organic raw milk up to the maximum level of 50 mL (approximately 1750 mg protein). Only one child could tolerate the shop milk to this level, but in all other cases a lower amount of shop milk was tolerated, and the provocation had to be stopped due to the development of allergic symptoms. On average, children could only tolerate 8.6 ± 5.3 mL shop milk.

Table 1. Organic raw cow's milk tolerated by cow's milk allergic children.

			Skin		Serum		DBPCT	
Patient	**Gender**	**Age (years)**	**SPT (mm)**	**APT (class)**	**Total IgE (kU/L)**	**Specific IgE (kU/L)**	**Raw milk (mL)**	**Shop milk (mL)**
1	M	2.65	10	++	322.0	26.3	50.0	2.0
2	M	3.52	4	++	123.0	4.2	50.0	10.0
3	M	0.55	7	+++	37.5	8.4	50.0	0.5
4	F	0.96	12	++	66.8	5.6	50.0	50.0
5	M	1.59	3	+++	nd	nd	50.0	1.0
6[#]	M	1.65	0	+	nd	nd	50.0	50.0
7[#]	M	1.09	0	+	nd	nd	50.0	50.0
8	M	0.96	5	++	98.6	12.4	50.0	0.5
9	F	0.83	7	+++	44.2	5.5	50.0	10.0
10	F	1.28	4	++	nd	nd	50.0	2.5
11	M	1.10	8	+++	nd	nd	50.0	1.0
Mean		**1.49**	**6.7**	**2.4**	**115.4**	**10.4**	**50.0**	**8.6**[**]
SEM		**0.32**	**1.0**	**0.2**	**43.4**	**3.4**	**0.0**	**5.3**

Shown are gender, age, skin prick test, atopy patch test, and serum total and cow's milk-specific IgE levels of 11 cow's milk allergic children before oral provocation as well as their level of tolerance to organic raw cow's milk and conventional shop milk during oral provocation. [#] Patient 6 and 7 were excluded from analysis since their milk allergy could not be confirmed in the skin prick test (0 mm). [**] $P < 0.01$ compared to raw milk tolerance level as analyzed with Wilcoxon signed-rank test. SPT, skin prick test; APT, atopy patch test; DBPCT, double-blind placebo-controlled trial; nd, not determined.

DISCUSSION

In this study, we demonstrate that raw (unprocessed) cow's milk has a lower allergenic potential than processed cow's milk. Similar results were observed when only looking at the whey protein fraction of the milk, suggesting that this fraction contributes to the observed differences. The preclinical evidence was supported by a proof-of-concept provocation pilot in which cow's milk allergic children could tolerate raw cow's milk but not commercially available processed milk. These results provide evidence that milk processing negatively influences the allergenicity of milk.

Today's Western society mainly consumes processed milk. Processed milk is safe in terms of pathogens, and the extended shelf life makes it easy to consume in everyday life. However, milk processing also induces unwanted changes in the milk composition. Proteins with potential beneficial health properties (partly) lose functionality, and the context of allergen presentation to the immune system may be altered (3-5, 15). Current literature mostly demonstrates the adverse effect of milk processing on allergic diseases like asthma and atopy (7, 12, 13). Milk processing, especially heating, abolishes the allergy-protective effects observed after consumption of raw cow's milk. This loss of protection is often attributed to the denaturation, and subsequent loss of functionality of immunomodulatory proteins present in the whey fraction of the milk (12, 14, 15). However, whether milk processing also affects the allergenicity of the milk is barely researched.

The term allergenicity or allergenic potential is defined in literature as 'the potential of a material to cause sensitization and allergic reactions, frequently associated with IgE antibody' (22). The allergenicity of milk is thus determined by two factors; the sensitizing capacity of the milk and the capacity of the milk to bind to IgE antibodies and thereby inducing an allergic reaction. Both factors have been investigated in this study.

When looking at sensitization, our data show that milk processing increases the sensitizing capacity of cow's milk. In a murine model, we observed less acute allergic symptoms in mice sensitized to raw milk compared to mice sensitized to processed milk. Next to reduced acute allergic symptoms, also allergen-specific IgE levels and Th2 cytokine concentrations were inhibited. The effects seemed to be dependent on processing time and temperature. The strongest sensitizing capacity was observed for heated raw milk which was heated for 10 min at 80 °C. The shop milk, heated for 15 s at 73 °C (pasteurization), was less allergenic. However, we should be careful with drawing this conclusion since the shop milk was besides pasteurized also homogenized and was furthermore derived from another milk source. Reduced allergic sensitization was also observed when only looking at the whey protein fraction of the milk. In our murine model, native, raw milk-derived, whey proteins induced a lower allergic response than heated, processed milk-derived, whey proteins.

Allergic sensitization can be influenced by many factors, like host genotype, type of allergen, amount, frequency, and route of allergen exposure but also whether allergen exposure occurs in combination with components that enhance/reduce the sensitization (23). In addition, milk processing can affect the sensitizing capacity by inducing structural and chemical alterations in milk proteins. Denaturation, aggregation, and the Maillard reaction with other molecules, like sugars, are some examples that can have an effect on the sensitizing capacity (24). Unfortunately, little is known about these topics. Few, if any, studies have examined the effect of milk processing on the sensitizing capacity of whole milk *in vivo*. Roth-Walter *et al*. did investigate the effect of heating on the main milk allergens; casein, α-lactalbumin, and β-lactoglobulin (16). In a murine model, they showed that the whey proteins, α-lactalbumin and β-lactoglobulin, form aggregates upon heating and that these aggregates enhanced allergic sensitization, as evidenced by increased IgE and Th2 cytokine responses. Since caseins naturally form micelles and thus already exist as aggregates, their sensitizing capacity was not affected by heat treatment. The enhanced sensitization by aggregated whey proteins was attributed to a shift in uptake from enterocytes to Peyer's patches, thereby increasing immunogenicity (16). We do not have data to confirm this shift in uptake but our study does confirm increased sensitization upon heat treatment. We observed this effect with whole milk and with the whey protein fraction, confirming that the effect is most likely independent of the caseins. Besides the formation of whey protein aggregates enhancing sensitization, there is also some evidence showing that the whey protein β-lactoglobulin presents some new epitopes upon heating (25). These epitopes can be uncovered by the unfolding of the protein upon heating or they can be created upon new chemical interactions. At the same time, it is also known that extensive heating can destroy epitopes (24). Whether the net effect is an increased sensitizing capacity has, to the authors knowledge, never been researched. Besides milk allergens, the whey protein fraction also contains a lot of immunomodulatory components. These components, like immunoglobulins, TGF-β, IL-10, lactoferrin, lysozyme, osteopontin, and lactoperoxidase, are known to enhance mucosal barrier function and to modulate the mucosal immune response. Together they might create an environment that favors unresponsiveness following allergen exposure (3, 14, 26). As most of these components are heat-sensitive, they (partly) lose functionality upon processing, providing another potential mechanism for the observed increase in sensitization.

Next to the effect on the sensitizing capacity, we determined whether native whey proteins also have a lower capacity to induce an allergic response when sensitization to heated whey proteins already occurred. We showed that native whey proteins indeed elicited a lower acute allergic skin response than heated whey proteins in heated whey protein-sensitized mice. This reduced acute allergic skin response coincided with a reduced Th2 cytokine response. Allergen-specific IgE levels were, however, not reduced in these mice, most likely because mice were sensitized to heated whey proteins so the IgE antibodies were already formed.

From clinical practice, it is known that patients can have IgE antibodies against a certain food allergen without having symptoms after exposure to that allergen (27). This indicates that factors other than IgE are playing an important role in the development of an allergic response. Such factors might be the concurrent presence of IgG antibodies, the presence of epithelial-derived mediators (e.g. galectin-9), the number and sensitivity of mast cells, the threshold of mast cells to induce IgE mediated activation but also the sensitivity of target organs to mast cell-derived mediators (28-30). We did not see differences in IgG levels (data not shown), and mucosal mast cell degranulation (mMCP-1) was not affected. However, since we observed a reduction in the acute allergic skin response there seems to be an effect on connective tissue mast cells. Mucosal and connective tissue mast cells were recently shown to underlie different symptoms of food allergy (31). The contribution of these mast cells in our model and whether raw milk and native whey proteins differently affect them should be clarified in future studies.

Another explanation for the lower capacity of native whey proteins to induce an allergic reaction in heated whey protein-sensitized mice is perhaps a difference in protein conformation which prevents IgE binding. However, in all experiments performed, we observed that IgE antibodies formed (whether this was against native or heated whey proteins) bound as well to native as to heated whey proteins. This suggests that our heat treatments did not induce major differences in protein conformation affecting B cell epitopes and hence IgE binding. The effect of milk processing on protein conformation and IgE-binding capacity is contradictory in current literature. Some studies report an increased IgE-binding capacity of α-lactalbumin and β-lactoglobulin heated at temperatures between 50 and 90 °C, while others showed a decrease (32-35). These effects were, however, observed in *in vitro* studies. Little *in vivo* research has been performed. There is some evidence showing that homogenized milk induces a stronger allergic reaction in milk allergic mice than raw milk (5, 36), but these findings could not be confirmed in clinical studies (37, 38).

The reduced allergenicity of raw milk was confirmed in a proof-of-concept provocation pilot. Cow's milk allergic children tolerated organic raw cow's milk up to the maximum level of 50 mL (approximately 1750 mg protein), whereas in most cases provocation with conventional shop milk had to be stopped earlier because of the development of allergic symptoms. To our knowledge, this is the first human pilot trial showing that traditional milk processing increases the allergenicity of raw cow's milk. Human trials have been performed with extensively heat-treated (baked) milk. These trials show that the majority of cow's milk allergic children tested could tolerate the extensively heated milk (39-41). This might indicate that the allergenicity of cow's milk is following a parabolic form, with a low allergenic potential at low (< 50 °C, e.g. raw cow's milk) and extremely high temperatures (> 180 °C, e.g. baked milk) and an increasing allergenic potential with temperatures in between. In the case of raw cow's milk, the lower allergenic potential could be caused by the fact that native, non-heated, proteins might be

taken up differently than aggregated proteins thereby reducing immunogenicity, and/or by immunomodulatory components present in raw cow's milk that might create an environment favoring unresponsiveness upon allergen exposure. In the case of baked milk, the lower allergenic potential could be caused by destruction of conformational epitopes (39).

The effect of the origin of the milk (organic vs conventional) on the allergenicity needs to be assessed in future studies. Different production and feeding methods on organic farms impact among others the fatty acid composition and antioxidant concentrations of the milk and might have contributed to the observed tolerance to organic raw milk in cow's milk allergic children (42, 43). In addition, the inclusion of children based on parent-reported cow's milk allergy potentially leading to a heterogeneous group of children (two children were left out because of the absence of a positive skin prick test), the limited number of children, the lack of IgE levels for some children, and the fact that the oral challenge did not include the full range of up to 3000 mg protein as recommended by the PRACTALL guidelines (44) represent the main limitations of this study.

In summary, in this study we demonstrated a lower allergenic potential of raw (unprocessed) cow's milk and native whey proteins as compared to their processed counterparts. These findings were extensively shown in a murine model and were confirmed in a human proof-of-concept provocation pilot. The observed effects were most likely not caused by an altered IgE binding. Instead, a change in allergen uptake and/or the formation of an environment favoring unresponsiveness upon allergen exposure might underlie the beneficial effects, although these are speculations which should be investigated in future studies. Risks from the certified, strictly controlled, raw milk used in this study are low, but a zero-risk can never be attained. The consumption of raw milk is therefore not recommended by the WHO. However, this study does add to the evidence on allergy-protective capacities of raw cow's milk and emphasizes once more the need for minimally processed milk. Besides, elucidating the raw milk components responsible for the allergy-protective effects and understanding the underlying mechanisms might help the development of new dietary concepts aimed at safe allergy management.

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SUPPLEMENTARY FIGURES

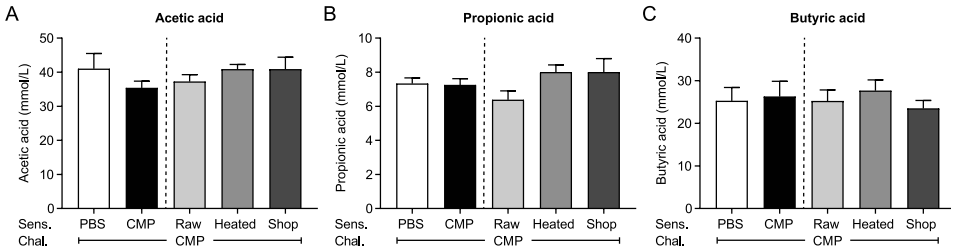


Figure S1. No differences between groups in caecal short-chain fatty acid concentrations. (**A**) Acetic acid, (**B**), propionic acid, and (**C**) butyric acid concentrations measured in caecum content on day 34, as previously described (45). Data are presented as mean ± SEM, *n* = 6 in PBS group and *n* = 8 in all other groups. No significant differences were observed. Sens., sensitization; chal., challenge; CMP, cow's milk protein; raw, raw cow's milk; heated, heated raw cow's milk; shop, shop milk.

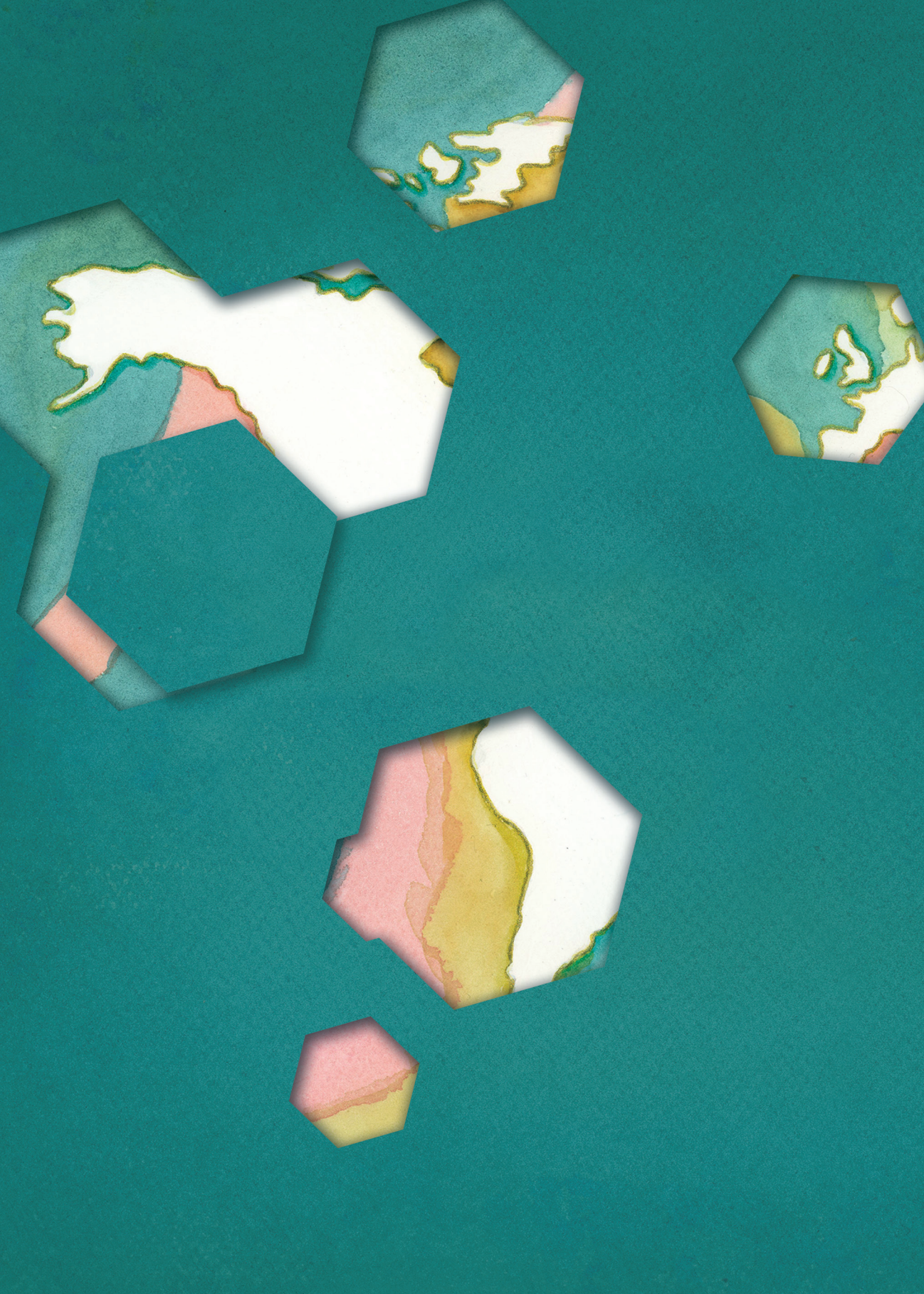
REFERENCES

1. Steele JH. History, trends, and extent of pasteurization. *J Am Vet Med Assoc* (2000) 217(2): 175-8.
2. Oliver SP, Boor KJ, Murphy SC, and Murinda SE. Food safety hazards associated with consumption of raw milk. *Foodborne Pathog Dis* (2009) 6(7): 793-806.
3. Chatterton DE, Nguyen DN, Bering SB, and Sangild PT. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int J Biochem Cell Biol* (2013) 45(8): 1730-47.
4. Michalski MC and Januel C. Does homogenization affect the human health properties of cow's milk? *Trends Food Sci Tech* (2006) 17(8): 423-37.
5. Poulsen OM, Hau J, and Kollerup J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. *Clin Allergy* (1987) 17(5): 449-58.
6. Kusche D, Sahm H, and Baars T. Konsum ökologischer Milch aus gesundheitlichen Gründen-Eine qualitative Erhebung auf deutschen Demeter Milchviehbetrieben und bei ihren Kunden. (2009).
7. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
8. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
9. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, *et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
10. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
11. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
12. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T, *et al*. Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model. *Front Immunol* (2017) 8: 1045.
13. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-1706 e13.
14. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
15. Brick T, Ege M, Boeren S, Bock A, von Mutius E, Vervoort J, *et al*. Effect of processing intensity on immunologically active bovine milk serum proteins. *Nutrients* (2017) 9(9): 963.
16. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J, *et al*. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy* (2008) 63(7): 882-90.
17. Bundesministerium der Justiz und für Verbraucherschutz. Verordnung über die Güteprüfung und Bezahlung der Anlieferungsmilch (Milch-Güteverordnung). (1980) Available from: http://www.gesetze-im-internet.de/milchg_v/index.html Accessed on: January 9 2019

18. van Esch BCAM, van Bilsen JH, Jeurink PV, Garssen J, Penninks AH, Smit JJ*, et al*. 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(1): 95-102.
19. Li XM, Schofield BH, Huang CK, Kleiner GI, and Sampson HA. A murine model of IgE-mediated cow's milk hypersensitivity. *J Allergy Clin Immunol* (1999) 103(2 Pt 1): 206-14.
20. Schouten B, van Esch BC, Hofman GA, van den Elsen LW, Willemsen LE, and Garssen J. Acute allergic skin reactions and intestinal contractility changes in mice orally sensitized against casein or whey. *Int Arch Allergy Immunol* (2008) 147(2): 125-34.
21. Turjanmaa K, Darsow U, Niggemann B, Rance F, Vanto T, and Werfel T. EAACI/GA2LEN position paper: present status of the atopy patch test. *Allergy* (2006) 61(12): 1377-84.
22. Verhoeckx KC, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S*, et al*. Food processing and allergenicity. *Food Chem Toxicol* (2015) 80: 223-40.
23. Galli SJ, Tsai M, and Piliponsky AM. The development of allergic inflammation. *Nature* (2008) 454(7203): 445-54.
24. Bu G, Luo Y, Chen F, Liu K, and Zhu T. Milk processing as a tool to reduce cow's milk allergenicity: a mini-review. *Dairy Sci Technol* (2013) 93(3): 211-23.
25. Davis PJ and Williams SC. Protein modification by thermal processing. *Allergy* (1998) 53(46 Suppl): 102-5.
26. Perdijk O, van Splunter M, Savelkoul HFJ, Brugman S, and van Neerven RJJ. Cow's milk and immune function in the respiratory tract: potential mechanisms. *Front Immunol* (2018) 9: 143.
27. Sampson HA, Aceves S, Bock SA, James J, Jones S, Lang D*, et al*. Food allergy: a practice parameter update-2014. *J Allergy Clin Immunol* (2014) 134(5): 1016-25 e43.
28. Renz H, Allen KJ, Sicherer SH, Sampson HA, Lack G, Beyer K*, et al*. Food allergy. *Nat Rev Dis Primers* (2018) 4: 17098.
29. de Kivit S, Saeland E, Kraneveld AD, van de Kant HJ, Schouten B, van Esch BCAM*, et al*. Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans. *Allergy* (2012) 67(3): 343-52.
30. Niki T, Tsutsui S, Hirose S, Aradono S, Sugimoto Y, Takeshita K*, et al*. Galectin-9 is a high affinity IgE-binding lectin with anti-allergic effect by blocking IgE-antigen complex formation. *J Biol Chem* (2009) 284(47): 32344-52.
31. Benede S and Berin MC. Mast cell heterogeneity underlies different manifestations of food allergy in mice. *PLoS One* (2018) 13(1): e0190453.
32. Bu G, Luo Y, Zheng Z, and Zheng H. Effect of heat treatment on the antigenicity of bovine α-lactalbumin and β-lactoglobulin in whey protein isolate. *Food Agr Immunol* (2009) 20(3): 195-206.
33. Ehn BM, Ekstrand B, Bengtsson U, and Ahlstedt S. Modification of IgE binding during heat processing of the cow's milk allergen beta-lactoglobulin. *J Agric Food Chem* (2004) 52(5): 1398-403.
34. Taheri-Kafrani A, Gaudin JC, Rabesona H, Nioi C, Agarwal D, Drouet M*, et al*. Effects of heating and glycation of beta-lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. *J Agric Food Chem* (2009) 57(11): 4974-82.
35. Kleber N, Krause I, Illgner S, and Hinrichs J. The antigenic response of beta-lactoglobulin is modulated by thermally induced aggregation. *Eur Food Res Technol* (2004) 219(2): 105-10.
36. Poulsen OM, Nielsen BR, Basse A, and Hau J. Comparison of intestinal anaphylactic reactions in sensitized mice challenged with untreated bovine milk and homogenized bovine milk. *Allergy* (1990) 45(5): 321-6.
37. Pelto L, Rantakokko HK, Lilius EM, Nuutila J, and Salminen S. No difference in symptoms and receptor expression in lactose-intolerant

and in milk-hypersensitive subjects following intake of homogenized and unhomogenized milk. *Int Dairy J* (2000) 10(11): 799-803.

38. Host A and Samuelsson EG. Allergic reactions to raw, pasteurized, and homogenized/ pasteurized cow milk: a comparison. A double-blind placebo-controlled study in milk allergic children. *Allergy* (1988) 43(2): 113-8.
39. Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, *et al*. Tolerance to extensively heated milk in children with cow's milk allergy. *J Allergy Clin Immunol* (2008) 122(2): 342-7 e1-2.
40. Mehr S, Turner PJ, Joshi P, Wong M, and Campbell DE. Safety and clinical predictors of reacting to extensively heated cow's milk challenge in cow's milk-allergic children. *Ann Allergy Asthma Immunol* (2014) 113(4): 425-9.
41. Caubet JC, Nowak-Wegrzyn A, Moshier E, Godbold J, Wang J, and Sampson HA. Utility of casein-specific IgE levels in predicting reactivity to baked milk. *J Allergy Clin Immunol* (2013) 131(1): 222-4 e1-4.
42. Kusche D, Kuhnt K, Ruebesam K, Rohrer C, Nierop AF, Jahreis G, *et al*. Fatty acid profiles and antioxidants of organic and conventional milk from low- and high-input systems during outdoor period. *J Sci Food Agric* (2015) 95(3): 529-39.
43. Srednicka-Tober D, Baranski M, Seal CJ, Sanderson R, Benbrook C, Steinshamn H, *et al*. Higher PUFA and n-3 PUFA, conjugated linoleic acid, alpha-tocopherol and iron, but lower iodine and selenium concentrations in organic milk: a systematic literature review and meta- and redundancy analyses. *Br J Nutr* (2016) 115(6): 1043-60.
44. Sampson HA, Gerth van Wijk R, Bindslev-Jensen C, Sicherer S, Teuber SS, Burks AW, *et al*. Standardizing double-blind, placebo-controlled oral food challenges: American Academy of Allergy, Asthma & Immunology-European Academy of Allergy and Clinical Immunology PRACTALL consensus report. *J Allergy Clin Immunol* (2012) 130(6): 1260-74.
45. Bakker-Zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJ, and Bindels JG. 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(5): 783-90.



CHAPTER 9

Direct inhibition of the allergic effector response by raw cow's milk – An extensive *in vitro* assessment

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ABSTRACT

The mechanisms underlying the allergy-protective effects of raw cow's milk are poorly understood. The current focus is mainly on modulation of T cell responses. In the present study, we investigated whether raw cow's milk can also directly inhibit mast cells, the key effector cells in IgE-mediated allergic responses. Primary murine bone marrow-derived mast cells (BMMC) and peritoneal mast cells (PMC), were incubated with raw milk, heated raw milk, or shop milk prior to IgE-mediated activation. Effects on mast cell activation and underlying signaling events were assessed. Raw milk was furthermore fractionated based on molecular size and obtained fractions were tested for their capacity to reduce IgE-mediated mast cell activation. Coincubation of BMMC and PMC with raw milk prior to activation reduced β-hexosaminidase release and IL-6 and IL-13 production, while heated raw milk or shop milk had no effect. The reduced mast cell activation coincided with a reduced intracellular calcium influx and reduced ERK phosphorylation, both downstream signaling events of the FcεRI. In addition, raw milk reduced the translocation of engaged FcεRI complexes into lipid rafts. Raw milk fractionation showed that the heat-sensitive raw milk components responsible for the reduced mast cell activation are likely to have a molecular weight of > 37 kDa. The present study demonstrates that raw cow's milk can also directly affect mast cell activation. These results extend the current knowledge on mechanisms via which raw cow's milk prevents allergic diseases, which is crucial for the development of new nutritional strategies to reduce allergic diseases.

INTRODUCTION

Mast cells are granular immune cells crucial in type I hypersensitivity reactions. IgE-mediated allergic reactions are induced when allergens cross-link allergen-specific IgE antibodies bound to high-affinity IgE receptors (FcεRI) expressed on the mast cell surface (1, 2). This cross-linking causes aggregation of the FcεRI, which triggers a cascade of intracellular signaling pathways resulting in mast cell activation (3). Upon activation, mast cells secrete various soluble mediators generally divided in three categories; preformed mediators stored in the cell's cytoplasmic granules (e.g. histamine, heparin, proteases), *de novo* generated lipid mediators (e.g. prostaglandins, leukotrienes, platelet-activating factor), and *de novo* synthesized cytokines and chemokines (e.g. TNFα, IL-6) (4). By inducing vasodilation, vascular permeability, smooth muscle contraction, and mucus secretion, these mediators are responsible for the initiation and exacerbation of allergic symptoms (3).

Given the pivotal role of mast cells in allergic diseases, inhibition of IgE-mediated mast cell activation is a common therapeutic strategy. Several pharmacological agents have been developed to block mast cell mediator receptors on target cells (e.g. antihistamines), to inhibit mediator synthesis (e.g. steroids, nonsteroidal anti-inflammatory drugs), and to prevent IgE-driven mast cell activation (e.g. Omalizumab) (2). However, these drugs have varying success rates making strict allergen avoidance still the only effective treatment (5).

Another approach is to reduce allergic sensitization and to favor immune tolerance. For example, by promoting aspects of lifestyle that seem to reduce the risk of allergic diseases based on epidemiological studies. The consumption of raw, unprocessed, cow's milk is such an aspect. Epidemiological studies have consistently shown an inverse association between raw cow's milk consumption and the development of allergic diseases (6-10). These findings were strengthened by causal evidence showing the potency of raw cow's milk to reduce/prevent allergic diseases (11-13). As source of immunomodulatory components, raw cow's milk is speculated to exert its allergy-protective effects by creating a tolerogenic environment favoring unresponsiveness upon allergen exposure (14-16). Recently, we provided evidence to support this hypothesis by demonstrating in a murine model for food allergy that the suppression of allergic symptoms by raw cow's milk was accompanied by a reduction in allergen-specific Th2 cell responsiveness and an induction of tolerance-associated cell types, like $CD103^+$ dendritic cells and regulatory T cells (12).

Currently, the allergy-protective effects of raw cow's milk are therefore mainly attributed to the capacity of the milk to modulate T cell responses. However, the key role of mast cells in allergic diseases raises the question whether raw cow's milk can also directly influence the allergic effector response by targeting mast cell activation and function. To investigate this, effects

on *in vitro* IgE-mediated mast cell activation were studied. Since there is increasing evidence showing that the protective effects of raw cow's milk are abolished upon milk processing (6, 11, 17), modulation by heated raw milk and shop milk (store-bought milk) was also assessed. In addition, raw milk was fractionated based on size and effects of raw milk fractions on mast cell activation were evaluated to gain more insight into the components contributing to the protection against allergic diseases.

MATERIALS & METHODS

Isolation and culture of primary mouse mast cells

Primary mast cells were generated from naïve female C3H/HeOuJ mice. To obtain bone marrow-derived mast cells (BMMC), as representatives of mucosal-type mast cells, femurs and tibiae were removed and thoroughly flushed with culture medium (RPMI 1640 medium [Lonza, Verviers, Belgium] supplemented with 10% fetal bovine serum [FBS; Bodinco, Alkmaar, The Netherlands], 100 U/mL penicillin and 100 µg/mL streptomycin [Sigma-Aldrich, Zwijndrecht, The Netherlands], 20 mM Hepes, 0.1 mM MEM non-essential amino acids, 2 mM GlutaMAX, 1 mM sodium pyruvate and 50 µM 2-mercaptoethanol [all purchased from Gibco, Thermo Fisher Scientific, Paisley, Scotland]) by using a needle and syringe. The suspension of bone marrow cells was passed through a 70 µm nylon cell strainer, centrifuged (1400 rpm, 6 min), and incubated with hypotonic lysis buffer (8.3 g NH_4Cl, 1 g KHC_3O, and 37.2 mg EDTA dissolved in 1 L demi water, filter sterilized) to remove red blood cells. Cell suspensions were subsequently resuspended in freezing medium consisting of 40% culture medium, 10% dimethyl sulphoxide (DMSO; Sigma-Aldrich) and 50% FBS and stored in liquid nitrogen until culture. For each culture, bone marrow cells were thawed and cultured in culture medium supplemented with 20 ng/mL IL-3 and stem cell factor (SCF; ProSpec, Ness-Ziona, Israel) at 37 °C. Subsequently, half of the culture medium was refreshed weekly, supplemented with 10 ng/mL IL-3 and SCF. Cell density was kept at 1-1.5 × 10^6 cells/mL. BMMC were used for experiments after 4 to 8 weeks of culturing. Peritoneal mast cells (PMC), as representatives of connective tissue-type mast cells, were generated as follows; the peritoneal cavity was washed with cold PBS and cells were collected. After lysing red blood cells, cells were cultured in culture medium supplemented with 50 ng/mL IL-3, IL-4, and SCF (ProSpec). After 1 week of culturing, nonadherent cells were collected and cultured in fresh culture medium (18). Subsequently, half of the culture medium was refreshed twice a week. PMC were used for experiments after 4 weeks of culturing.

Milk types

The raw cow's milk used was collected from a biodynamic dairy farm (Hof Dannwisch, Horst, Germany). Upon collection, part of the raw milk was aliquoted and stored at -20 °C until further use. The remainder was heated in a water bath for 10 min at 80 °C, cooled to room

temperature, aliquoted, and then stored at -20 °C until further use (heated raw milk). The shop milk used was a pasteurized and homogenized milk standardized at 3.8% fat (EDEKA, Germany). To keep treatments equal, this milk was also aliquoted and stored at -20 °C. The day before experiments were conducted, the different milk types were put overnight in the fridge to keep the thawing process constant. Just before use, they were placed in a water bath for 30 min at 37 °C to adjust the temperature to culturing conditions and to obtain homogeneous solutions.

Milk fractions

To fractionate raw cow's milk based on molecular size, qEV size exclusion columns (Izon Science, Oxford, UK) were used according to the manufacturer's protocol. Briefly, 0.5 mL of 10,000 *g* raw milk supernatant (free of cells, cell debris, and cream) was loaded onto the size exclusion column (Izon Science) and the first 3 mL of eluent was discarded. Eluent fractions of 0.5 mL were then collected up to 12 mL (24 fractions) by continuously adding RPMI 1640 medium without L-glutamine and phenol red (Lonza) to the column. Protein content of each fraction was quantified by using a NanoDrop ND-1000 spectrophotometer (A280; Thermo Fisher Scientific). To determine the molecular weight of the proteins in each fraction, proteins were separated by using a 12.5% SDS-PAGE under non-reducing conditions and visualized with SYPRO® Ruby Protein Gel Stain (Bio-Rad, Veenendaal, The Netherlands). Fractions were stored at -80 °C until further use.

Mast cell activation assay

BMMC (1×10^6 cells/mL) and PMC (3.2×10^5 cells/mL) were incubated overnight with 5% v/v raw milk, heated raw milk, or shop milk at 37 °C. After washing 3 times with assay medium (RPMI 1640 medium without L-glutamine and phenol red [Lonza], supplemented with 1% FBS [Bodinco] and 2 mM GlutaMAX [Gibco, Thermo Fisher Scientific]), cells were primed with 10-20% v/v 2,4-dinitrophenol (DNP)-specific IgE (culture supernatant of IgE-producing hybridoma cells, clone 26.82) for 1 h at 37 °C. Subsequently, cells were washed twice and stimulated by a range of DNP-HSA (DNP conjugated to human serum albumin; Sigma-Aldrich) concentrations (BMMC: 0-100 ng/mL; PMC: 0-12.5 ng/mL) for 1 h at 37 °C. In addition, BMMC were also stimulated by a range of rat anti-mouse IgE mAb concentrations (BD Biosciences, Alphen aan de Rijn, The Netherlands; 0-125 ng/mL) and by ionomycin (1 µM; Sigma-Aldrich). The magnitude of mast cell activation was determined by measuring β-hexosaminidase (β-hex) and cytokine release. B-hex release was quantified by measuring fluorescence (excitation 350 nm/emission 460 nm) with a Fluoroskan Ascent® Microplate Fluorometer (Thermo Fisher Scientific) after incubating cell-free supernatant with 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4-MUG; 158 µM; Sigma-Aldrich) in citrate buffer (0.1 M, pH 4.5; Acros Organics, Geel, Belgium) for 1 h at 37 °C and terminating the enzymatic reaction by adding glycine buffer (0.1 M, pH 10.7; Merck, Darmstadt, Germany). Maximum β-hex release was determined by lysing the cells with

0.5% Triton X-100 (Sigma-Aldrich). The percentage of β-hex release was calculated using the following formula: (A-B)/(T-B) × 100%, where A is the amount of β-hex released from stimulated cells, B the amount released from unstimulated cells and T the amount of β-hex released from Triton X-100 lysed cells. Cytokine production was determined in cell-free supernatant harvested 18 h after DNP-HSA stimulation. IL-6 concentrations were analyzed using a mouse IL-6 ELISA MAX Standard Set (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. IL-13 concentrations were analyzed by means of ELISA as described elsewhere (19). For raw milk fractions, BMMC were incubated for 2 h with the fractions, supplemented with 10% bovine serum albumin (BSA; Sigma-Aldrich) and 1% GlutaMAX. Mast cell activation was performed as described above.

Flow cytometric analysis of BMMC

After overnight milk incubation, BMMC were washed and incubated with anti-mouse CD16/CD32 (Mouse BD Fc Block; BD Biosciences) for 15 min on ice to block non-specific binding sites. Cells were subsequently stained with FcεRI-PE-Cy7 and CD117-APC antibodies (Thermo Fisher Scientific) for 45 min on ice. Viable cells were distinguished using the dead cell dye YO-PRO®-1 Iodide according to manufacturer's instructions (Thermo Fisher Scientific). Cell viability and expression of FcεRI and CD117 were measured on the FACS Canto II (BD Biosciences) and analyzed with FlowLogic Software (Inivai Technologies, Mentone, Australia). Isotype controls were used and cut-off gates for positivity were determined with fluorescence-minus-one controls.

Analysis of calcium flux

BMMC were incubated overnight with the different milk types, washed, and primed for 1 h with 10-20% v/v DNP-specific IgE (culture supernatant of IgE-producing hybridoma cells, clone 26.82) as described above. Cells were then washed again and loaded with the calcium-sensitive dye Fluo-4, AM (4 μM; Invitrogen, Thermo Fisher Scientific) by incubating them at 37 °C for 30 min, followed by 30 min at room temperature. After Fluo-4, AM loading, cells were washed and incubated for 30 min at room temperature with RPMI 1640 medium (without L-glutamine and phenol red; Lonza)/1% FBS (Bodinco). Prior to stimulation with DNP-HSA, baseline fluorescent readings were measured in 4 s intervals for 1 min using a Fluoroskan Ascent® Microplate Fluorometer with 492 nm excitation and 518 nm emission filters (Thermo Fisher Scientific). Cells were then treated with DNP-HSA (12.5 ng/mL; Sigma-Aldrich) or RPMI 1640 medium/1% FBS (as a control) and fluorescence was measured in 10 s intervals for 7 min.

Immunoblotting for membrane-bound IgE expression and ERK phosphorylation

For determination of membrane-bound IgE expression, BMMC were lysed for 15 min on ice with PBS/0.5% Triton X-100 (Sigma-Aldrich) buffer supplemented with protease inhibitors (cOmplete, Mini Protease Inhibitor Cocktail; Roche Diagnostics, Mannheim, Germany) after

incubation with the different milk types and IgE-mediated activation as described earlier. After centrifugation for 10 min at 4000 *g*, supernatant was collected, and SDS sample loading buffer (58.3 mM Tris-HCl (pH 6.8), 6% v/v glycerol, 1.7% w/v SDS, 0.01% w/v bromophenol blue, and 100 mM DTT) was added. For determination of extracellular signal-regulated kinase (ERK) phosphorylation, BMMC were incubated for 3 h at 37 °C with RMPI 1640 medium (without L-glutamine and phenol red; Lonza)/0.2% BSA (Sigma-Aldrich) after milk incubation and IgE priming, to deplete them from serum. Cells were subsequently washed in PIPES buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl, 5.6 mM glucose, 10 mM PIPES and 1.4 mM $CaCl_2 \cdot 2H_2O$) and stimulated for 10 min at 37 °C by DNP-HSA (0-50 ng/mL; Sigma-Aldrich) diluted in PIPES buffer. Phosphorylation was stopped and cells were lysed by adding SDS sample loading buffer supplemented with protease inhibitors (cOmplete, Mini Protease Inhibitor Cocktail; Roche Diagnostics), phosphatase inhibitors (phosSTOP; Roche Diagnostics), 5U benzonase nuclease (Merck), 1 mM $MgCl_2$, and 0.5 mM AEBSF (Merck). Upon addition of loading buffer, samples (for both protocols) were boiled for 5 min and loaded onto a 4-20% precast polyacrylamide gel (Bio-Rad). After running the gels, proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad) and non-specific binding was blocked by incubation with TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat dry milk for 1 h at room temperature. Membranes were then incubated with either HRP-conjugated goat anti-mouse IgE antibody (SouthernBiotech, Birmingham, AL, USA) in TBS-T/5% milk for 1 h at room temperature or rabbit anti-mouse phospho-p44/42 MAPK antibody (ERK1/2; Cell Signaling Technology, Leiden, The Netherlands) in TBS-T/5% BSA overnight at 4 °C. The membrane detecting ERK phosphorylation was subsequently incubated with HRP-conjugated goat anti-rabbit antibody (DAKO, Eindhoven, The Netherlands) in TBS-T/5% milk for 1 at room temperature. HRP-detected protein bands were visualized using Clarity Western ECL Substrate (Bio-Rad) and imaged and quantified by ChemiDoc MP Imaging System (Bio-Rad) and Image Lab Software (version 5.2; Bio-Rad). The expression of IgE and phosphorylated ERK (pERK) was normalized using mouse β-actin (Cell Signaling Technology).

Statistical analysis

Results are expressed as mean ± SEM and differences compared to the control group were statistically determined using one-way ANOVA followed by Dunnett's multiple comparisons test. For Western blot results, immunoblots and the corresponding densitometric values are displayed. To analyze the calcium flux, the area under the curve (AUC) was calculated. Results were considered statistically significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism software (version 7.03; GraphPad Software, San Diego, CA, USA).

RESULTS

Raw milk inhibits IgE-mediated mast cell activation

To determine whether raw cow's milk affects IgE-mediated mast cell activation, BMMC were sensitized with DNP-specific IgE and subsequently cross-linked with DNP-HSA to stimulate degranulation. As expected, DNP-HSA concentrations ranging from 0.78 to 50 ng/mL induced a dose-dependent increase in β-hex release (**Figure 1A**). Incubating BMMC overnight with raw milk prior to mast cell activation reduced the β-hex release by approximately 35% ($P < 0.05$; **Figure 1A**). Pre-treatment of BMMC with heated raw milk or shop milk had no effect (**Figure 1A**). Similar results were observed when anti-mouse IgE mAb was used to cross-link IgE bound to FcεRI to stimulate degranulation (data not shown). BMMC are considered to represent mucosal-type mast cells. The other main murine mast cell phenotype, the connective tissue-type mast cells, are represented by PMC (20). To assess whether raw milk exerts similar effects on both phenotypes, PMC were also activated in an allergen-specific manner. Because of their higher sensitivity, lower allergen concentrations were used (**Figure 1B**). Incubating PMC overnight with raw milk resulted in a comparable β-hex reduction of approximately 40% ($P < 0.05$; **Figure 1B**). Again, heated raw milk and shop milk showed no effect (**Figure 1B**). In addition, the effect of raw milk on *de novo* cytokine production upon mast cell activation (12.5 ng/mL DNP-HSA) was investigated. Consistent with the reduction in β-hex release, raw milk caused a 50% reduction in BMMC-produced IL-6 ($P = 0.0118$; **Figure 1C**). IL-13 concentrations were even reduced by 75% in raw milk-treated BMMC ($P = 0.0424$; **Figure 1D**). No inhibition in IL-6 and IL-13 production was observed after incubation with heated raw milk or shop milk. IgE-mediated stimulation of PMC did not result in detectable cytokine production (data not shown). In further experiments, BMMC were used to further delineate the mechanism of inhibition by raw milk.

Inhibition of IgE-mediated mast cell activation is not due to lower expression of FcεRI on mast cells or decreased viability

To confirm that the inhibition of IgE-mediated mast cell activation by raw milk was not due to lower receptor expression or reduced cell viability, FcεRI and CD117 expression and BMMC viability after overnight milk incubation were analyzed by flow cytometry. Untreated BMMC showed around 85% viability and approximately 90% of these cells expressed FcεRI and CD117 (**Figures 2A,B**). BMMC viability (data not shown) and FcεRI and CD117 expression were not affected by any of the milk types (**Figure 2B**).

Ionomycin-induced mast cell activation is not affected by raw milk exposure

In order to investigate whether raw milk incubation only reduced FcεRI-triggered mast cell activation, BMMC were activated with the calcium ionophore ionomycin. Ionomycin directly transports Ca^{2+} across the cell membrane and rapidly depletes intracellular calcium stores,

thereby it artificially increases intracellular calcium levels and bypasses the proximal signaling pathway of the FcεRI (21). In **Figure 2C**, it is shown that neither raw milk nor heated raw milk or shop milk significantly affected ionomycin-induced β-hex release, suggesting that raw milk may inhibit proximal steps of IgE-induced mast cell degranulation.

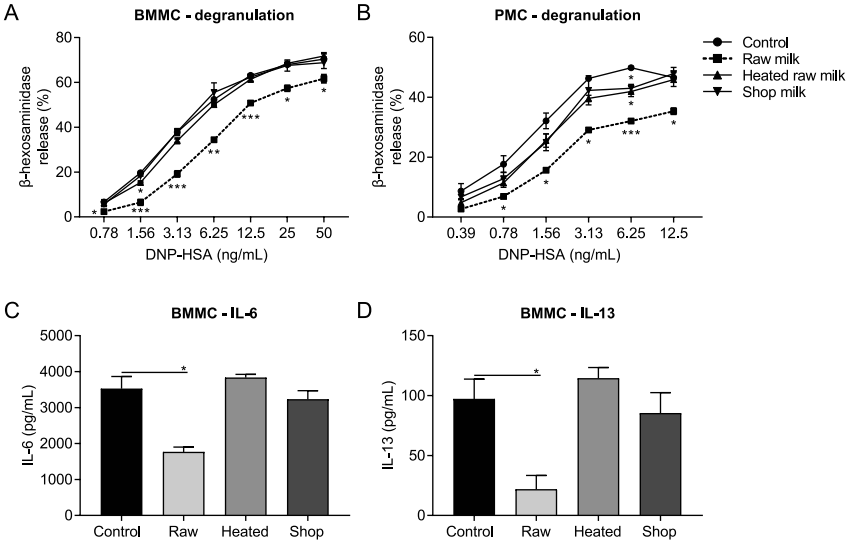

Figure 1. IgE-mediated mast cell activation reduced by raw milk. Primary mouse mast cells were incubated overnight with raw milk, heated raw milk, or shop milk before they were primed with DNP-specific IgE and stimulated by a range of DNP-HSA concentrations. β-hexosaminidase release by (**A**) BMMC and (**B**) PMC measured in supernatant collected 1 h after DNP-HSA stimulation. (**C**) IL-6 and (**D**) IL-13 production by BMMC measured in supernatant collected 18 h after DNP-HSA stimulation. Data are presented as mean ± SEM and are representative of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared to the control group as analyzed with one-way ANOVA followed by Dunnett's multiple comparisons test. BMMC, bone marrow-derived mast cells; PMC, peritoneal mast cells; DNP-HSA, 2,4-dinitrophenol conjugated to human serum albumin; raw, raw cow's milk; heated, heated raw cow's milk; shop, shop milk.

Raw milk-treated BMMC retain membrane-bound IgE expression after allergen-specific stimulation

To further understand the inhibitory effect of raw milk on FcεRI-induced mast cell activation, responses on parts of the associated signaling cascade were assessed. Upon allergen cross-linking, FcεRI complexes translocate into glycolipid-enriched membrane domains, better known as lipid rafts. This translocation is followed by rapid FcεRI internalization resulting in removal of the receptor from the cell membrane (22, 23). To investigate whether raw milk reduces IgE-mediated BMMC activation by preventing FcεRI migration to lipid rafts and subsequent internalization, membrane-bound IgE expression upon DNP-HSA stimulation was determined. As expected,

control BMMC showed a reduction in the presence of membrane-bound IgE with increasing DNP-HSA concentrations (**Figures 3A,B**). Interestingly, raw milk-treated BMMC retained IgE expression on their cell membrane after activation, regardless of DNP-HSA concentration (**Figures 3A,B**). Heated raw milk- and shop milk-exposed BMMC showed a similar reduction in membrane-bound IgE expression density as control BMMC (**Figures 3A,B**).

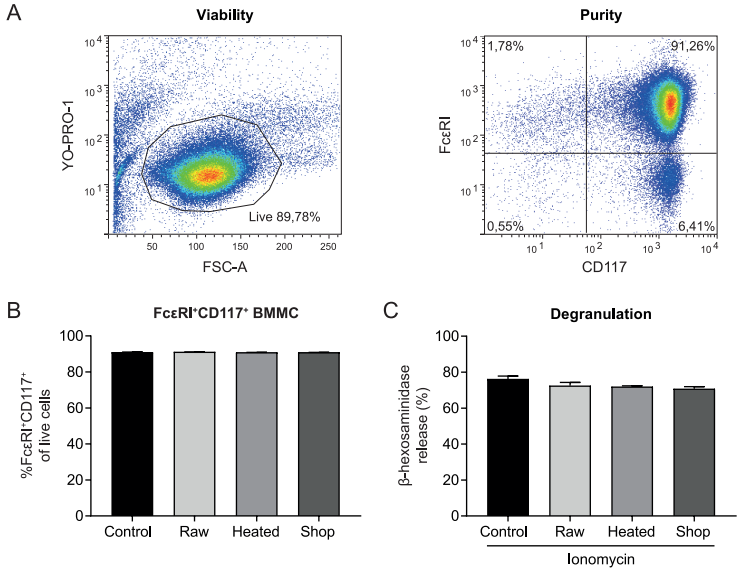

Figure 2. Raw milk exposure did not affect cell viability, FcεRI expression, and ionomycin-induced mast cell activation. (**A**) Viability and purity of cultured BMMC. (**B**) FcεRI and CD117 expression on BMMC after overnight milk exposure. (**C**) Ionomycin-induced BMMC activation after overnight milk exposure. Data are presented as mean ± SEM and are representative of three independent experiments. No significant differences were observed. FSC-A, forward scatter-area; BMMC, bone marrow-derived mast cells; raw, raw cow's milk; heated, heated raw cow's milk; shop, shop milk.

Decreased calcium influx upon allergen challenge in BMMC exposed to raw milk

Elevation of cytoplasmic calcium levels is key in the activation pathway leading to mast cell degranulation (3). To assess whether the inhibition of IgE-mediated BMMC activation by raw milk coincided with a reduced calcium influx, BMMC were loaded with the calcium-sensitive dye Fluo-4, and then exposed to DNP-HSA. Changes in Fluo-4 fluorescence, corresponding to changes in intracellular calcium levels, were subsequently examined. As shown in **Figure 4A**, control cells showed a rapid rise in intracellular calcium levels upon allergen challenge (12.5 ng/mL DNP-HSA). This increase peaked at about 60 s, gradually decreased thereafter, and tended to plateau after approximately 300 s (**Figure 4A**). No calcium influx was observed when control cells were not stimulated (**Figure 4A**). When BMMC were preincubated with raw milk,

the calcium influx was inhibited as shown by a significant reduction in the AUC (P = 0.0167; **Figure 4B**). BMMC preincubated with heated raw milk or shop milk showed similar intracellular calcium levels upon DNP-HSA challenge as control cells (**Figures 4A,B**).

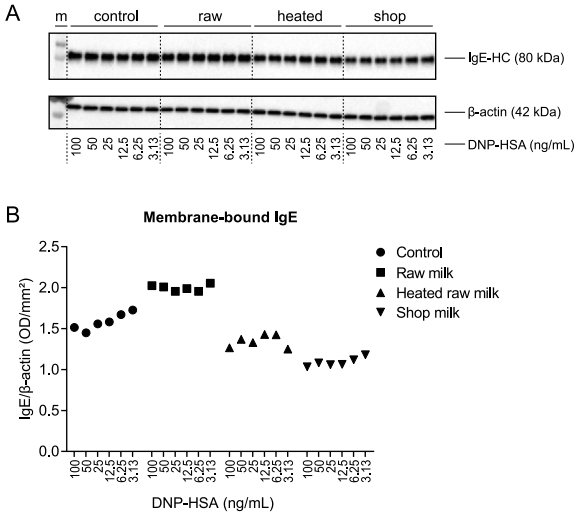

Figure 3. Membrane-bound IgE expression retained after DNP-HSA stimulation in raw milk-treated BMMC. After incubation with the different milk types and IgE-mediated activation by a range of DNP-HSA concentrations, BMMC were directly lysed in Triton X-100 for SDS-PAGE and immunoblotting. (**A**) Triton X-100 soluble fraction of BMMC lysates immunoblotted for IgE and β-actin. (**B**) Densitometric values of IgE normalized to β-actin. Results are representative of at least three independent experiments. M, marker; raw, raw cow's milk; heated, heated raw cow's milk; shop, shop milk; IgE-HC, IgE heavy chain; DNP-HSA, 2,4-dinitrophenol conjugated to human serum albumin; OD, optical density.

Reduced cytokine production after raw milk treatment coincided with lower ERK phosphorylation

Whereas an increase in intracellular calcium levels is crucial for mast cell degranulation, activation of the mitogen-activated protein kinase (MAPK) pathway is of importance for *de novo* synthesis and secretion of cytokines. The MAPK proteins ERK, p38, and c-Jun N-terminal kinase (JNK) regulate the phosphorylation of specific transcription factors important for the synthesis of cytokines (1). To examine whether the observed reduction in IL-6 and IL-13 production by raw milk-treated BMMC was the result of lower MAPK activation, ERK phosphorylation upon DNP-HSA stimulation was determined. Western blotting showed a lower ERK1/2 phosphorylation in the raw milk group compared to the control group (**Figures 5A,B**), which is in accordance with the reduction in cytokine production by raw milk-treated BMMC. For heated raw milk and shop milk, ERK1/2 phosphorylation levels were comparable to levels observed in untreated BMMC, which also corresponds to cytokine results (**Figures 5A,B**).

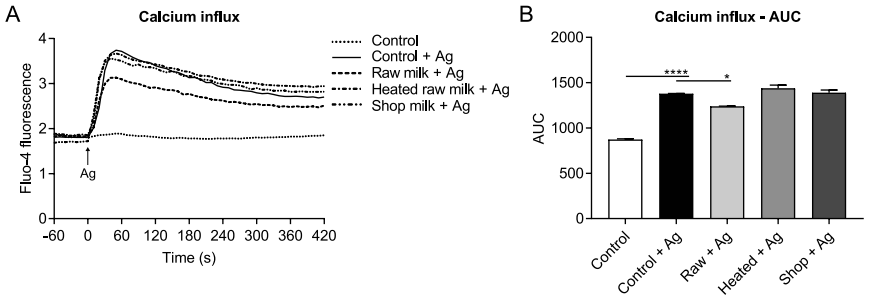

Figure 4. Raw milk-treated BMMC decreased calcium influx upon allergen challenge. (**A**) Changes in Fluo-4 fluorescence, representing changes in intracellular calcium levels, measured at baseline (for 60 s) and after allergen stimulation (indicated by the arrow; for 420 s). A representative graph is shown. (**B**) AUC analysis of the calcium influx, presented as mean ± SEM. Data are representative of three independent experiments. **P* < 0.05, *****P* < 0.0001, compared to the allergen-stimulated control group (control + Ag) as analyzed with one-way ANOVA followed by Dunnett's multiple comparisons test. Ag, allergen; control, unstimulated control BMMC; control + Ag, allergen-stimulated control BMMC; raw + Ag, allergen-stimulated raw milk-treated BMMC; heated + Ag, allergen-stimulated heated raw milk-treated BMMC; shop + Ag, allergen-stimulated shop milk-treated BMMC; AUC, area under the curve.

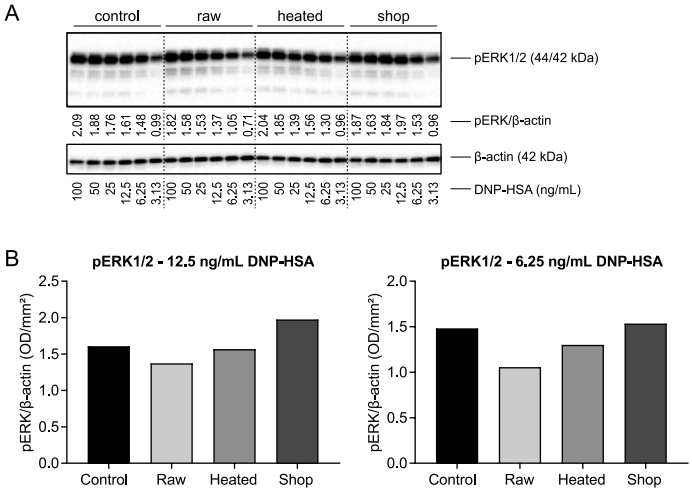

Figure 5. Lower ERK phosphorylation in BMMC treated with raw milk. After milk incubation and IgE priming, BMMC were incubated for 3 h without serum to reduce autophosphorylation. Cells were subsequently stimulated for 10 min with DNP-HSA after which phosphorylation was stopped and cells were lysed using SDS sample loading buffer. (**A**) Immunoblots for pERK and β-actin, including the densitometric values of the pERK/β-actin ratio at each DNP-HSA concentration. (**B**) Densitometric values of the pERK/β-actin ratio at 12.5 ng/mL DNP-HSA and 6.25 ng/mL DNP-HSA. Results are representative of three independent experiments. Raw, raw cow's milk; heated, heated raw cow's milk; shop, shop milk; pERK, phosphorylated ERK; DNP-HSA, 2,4-dinitrophenol conjugated to human serum albumin; OD, optical density.

Raw milk fractions 2 and 3 capable of reducing IgE-mediated mast cell activation

To gain more insight into the raw milk components responsible for the reduced mast cell activation, raw milk was fractionated based on molecular size and 24 raw milk fractions were tested for their capacity to reduce allergen-induced β-hex release. **Figure 6A** shows the chromatogram of proteins eluded from the gel-filtration column with a protein peak at fractions 2-5 and around fraction 18. Raw milk fractions were subsequently fractionated by SDS-PAGE and proteins were visualized using SYPRO® Ruby Protein Gel Stain to determine the molecular weight of the proteins in each fraction. Fractions 2-5 contained mostly proteins with a molecular weight between 50 and 75 kDa (**Figure 6B**). From fraction 11 onwards, smaller proteins with a molecular weight of around 10 to 25 kDa were more abundantly present (**Figure 6B**). When tested their effect on IgE-mediated mast cell activation, raw milk fractions 2 and 3 appeared to be the only fractions capable of reducing the allergen-induced β-release ($P = 0.0084$ and $P = 0.0794$, respectively; **Figure 6C**).

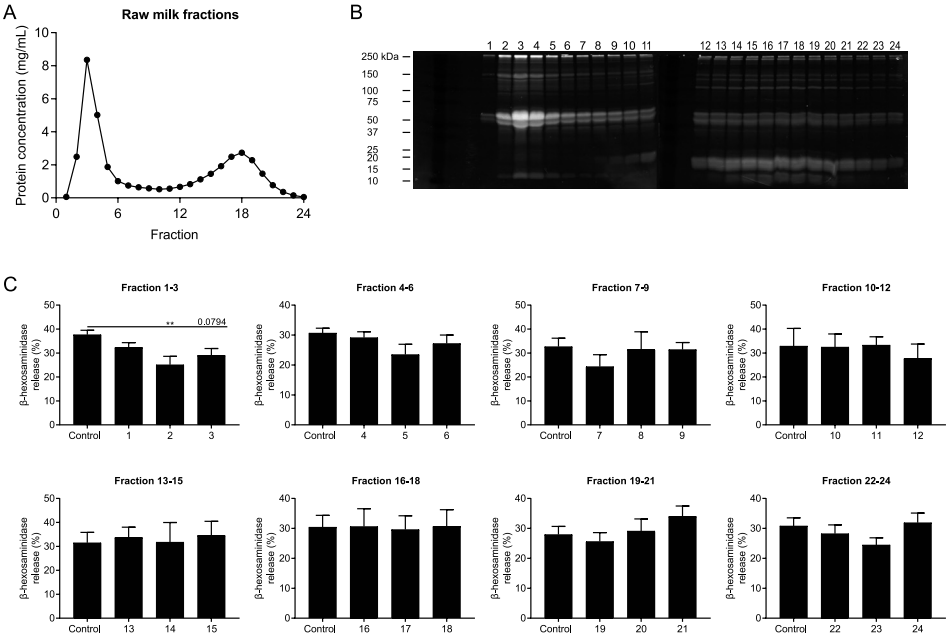

Figure 6. Reduction in IgE-mediated mast cell activation by raw milk fractions 2 and 3. (**A**) Chromatogram of proteins eluded from the gel-filtration column used to fractionate raw milk based on molecular size. (**B**) SYPRO® Ruby Protein Gel Stain of raw milk fractions loaded onto an SDS-PAGE. (**C**) β-hexosaminidase release by BMMC incubated with raw milk fractions prior to IgE-mediated activation. Results of the protein chromatogram and SYPRO® Ruby Protein Gel Stain are representative of three independent experiments. Results of the mast cell activation assay are presented as mean ± SEM of three independent experiments ($n = 3$). $^{**}P < 0.01$ compared to the control group as analyzed with one-way ANOVA followed by Dunnett's multiple comparisons test.

DISCUSSION

Previously, we observed that the suppression of local type 2 cytokine levels, produced by other immune cells than T cells, seemed to be crucial for the raw milk-induced prevention of allergic asthma in a murine house dust mite-induced asthma model (11). Since it is well known that type 2 cytokines can also be produced by mast cells and basophils (24, 25), this hinted towards a potential role of these effector cells in the allergy-protective effect. The present study therefore extensively investigated the direct effects of raw cow's milk on murine mast cells *in vitro*.

In this study, we demonstrate that raw cow's milk has the capacity to inhibit the allergic effector response *in vitro* by directly affecting mast cell activation. Exposing BMMC and PMC to raw milk prior to activation significantly reduced β-hex release and *de novo* IL-6 and IL-13 production. Since ionomycin-induced mast cell activation was not affected by raw milk, raw milk likely inhibits activation of the proximal signaling pathway of the FcεRI. Raw milk-treated mast cells furthermore retained membrane-bound IgE expression after allergen stimulation. This may indicate that raw milk exerts its effects by reducing the translocation of engaged FcεRI complexes into lipid rafts and subsequent receptor internalization, required for FcεRI-mediated mast cell responses. In line with previous epidemiological as well as preclinical studies showing a loss of allergy protection upon milk processing (6, 11-13, 17), heated raw milk and shop milk (store-bought milk) did not inhibit mast cell activation. Moreover, this study provides some insight into the raw milk components responsible for the reduced mast cell activation by demonstrating that these components are likely to have a molecular weight of > 37 kDa.

As we wanted to elaborate on previous findings observed in a murine allergic asthma model (11), murine mast cells were used. In mice, mucosal-type mast cells and connective tissue-type mast cells represent the two main mast cell phenotypes (20). BMMC and PMC are, respectively, considered as their *in vitro* equivalents. IgE-mediated BMMC activation was significantly inhibited by raw cow's milk, as illustrated by a reduced β-hex release and reduced *de novo* IL-6 and IL-13 production. The reduced BMMC activation coincided with reduced downstream signaling events of the FcεRI. Both intracellular calcium influx, key in the activation pathway leading to mast cell degranulation, and ERK phosphorylation, important for *de novo* synthesis and secretion of cytokines, were inhibited by raw milk. In PMC, a similar reduction in β-hex release was observed after IgE-mediated stimulation. However, IgE-mediated stimulation of PMC did not result in the secretion of newly formed cytokines (data not shown). This is in line with previous studies demonstrating that PMC secrete no or small amounts of newly formed proinflammatory mediators upon activation (18).

Interestingly, ionomycin-induced BMMC activation was not affected by raw milk. Since ionomycin artificially increases intracellular calcium levels and thereby bypasses the proximal signaling pathway of the FcεRI this suggests that raw milk probably acts on these earlier signaling events. Upon cross-linking of the FcεRI, the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domain of the β and γ receptor subunits are phosphorylated in a Lyn-dependent manner. The protein kinase Syk is then recruited to the phosphorylated ITAMs where it becomes activated, and subsequently phosphorylates and activates other proteins in the signaling cascade (3). Lipid rafts have been shown to play an important role in this early signaling process as they function as platforms capable of assembling the signal transduction machinery (26-28). Migration of the FcεRI to these lipid rafts upon allergen cross-linking has been demonstrated by several studies (22, 23). The fact that the FcεRI is soluble in Triton X-100 at steady state, but not after cross-linking (29) provides an opportunity to investigate FcεRI migration to lipid rafts upon allergen-specific stimulation. By immunoblotting the Triton X-100 soluble fraction of cell lysates for IgE, we could demonstrate a reduction in the presence of membrane-bound IgE with increasing DNP-HSA concentrations. This is in line with the previously mentioned studies (22, 23), indicating an increased migration of the FcεRI to lipid rafts upon allergen-specific stimulation. Interestingly, raw milk-treated BMMC retained IgE expression on their cell membrane after activation, regardless of DNP-HSA concentration. Retaining membrane-bound IgE expression, and thus preventing FcεRI migration to lipid rafts, might therefore be a way by which raw cow's milk was able to reduce IgE-mediated mast cell activation.

To gain more insight into the raw milk components responsible for the observed reduction in mast cell activation, raw milk was fractionated based on molecular size. From the 24 raw milk fractions obtained, only fraction 2 and 3 were able to reduce β-hex release in the mast cell activation assay. According to the SDS-PAGE, these fractions contained mostly proteins with a molecular weight between 50 and 75 kDa. Interestingly, the most abundant milk proteins, i.e. the caseins, β-lactoglobulin, and α-lactalbumin, all have a molecular weight of < 25 kDa. This indicates that these proteins are probably not responsible for the observed reduction in mast cell activation. In addition, this suggests that the observed effects are likely to be attributable to less abundant proteins with immunomodulatory functionalities. Characterization of the protective fractions by proteomic analysis might give more insight into the actual raw milk protein(s) involved.

In the *in vivo* situation, direct contact between mast cells and raw milk is unlikely. During passage through the gastrointestinal tract, raw milk will be, at least partly, degraded. Moreover, mast cells in the gut are located beneath the epithelial surface (30) which hinders direct interaction between raw milk and mast cells. However, for several raw milk components, such as lactoferrin and TGF-β, it has been demonstrated that they can survive passage through

the gastrointestinal tract upon ingestion (14). Whether these components can directly (e.g. due to epithelial barrier disruption as demonstrated in children with food allergy (31) or via transepithelial uptake) or indirectly (e.g. via modulation of epithelial cells or the gut microbiome) affect mast cells should be assessed in future studies.

In conclusion, we demonstrate a direct inhibition of the allergic effector response by raw cow's milk. Next to the already described capacity to modulate T cell responses, the present study shows that raw cow's milk is also able to directly influence mast cell activation. Mast cell activation was not affected by heated raw milk and shop milk, supporting the current evidence for a loss of allergy protection after milk processing and more specially, after heat treatment. By fractionating raw milk based on molecular size, we could demonstrate that the heat-sensitive raw milk components responsible for the reduced mast cell activation are likely to have a molecular weight of > 37 kDa. In addition, we showed that raw milk probably affects mast cell activation by acting on the proximal signaling pathway of the FcεRI. Raw milk-treated mast cells retained membrane-bound IgE expression, suggesting that raw milk reduced FcεRI migration to lipid rafts, crucial for FcεRI-mediated signal transduction. The evidence for a direct inhibition of mast cell activation by raw cow's milk, provided in this study, extends the current knowledge on mechanisms underlying the allergy-protective effects of raw cow's milk. Since raw cow's milk consumption is discouraged because of the possible contamination with pathogens, a better understanding of these mechanisms is key for the development of microbiologically safe alternatives.

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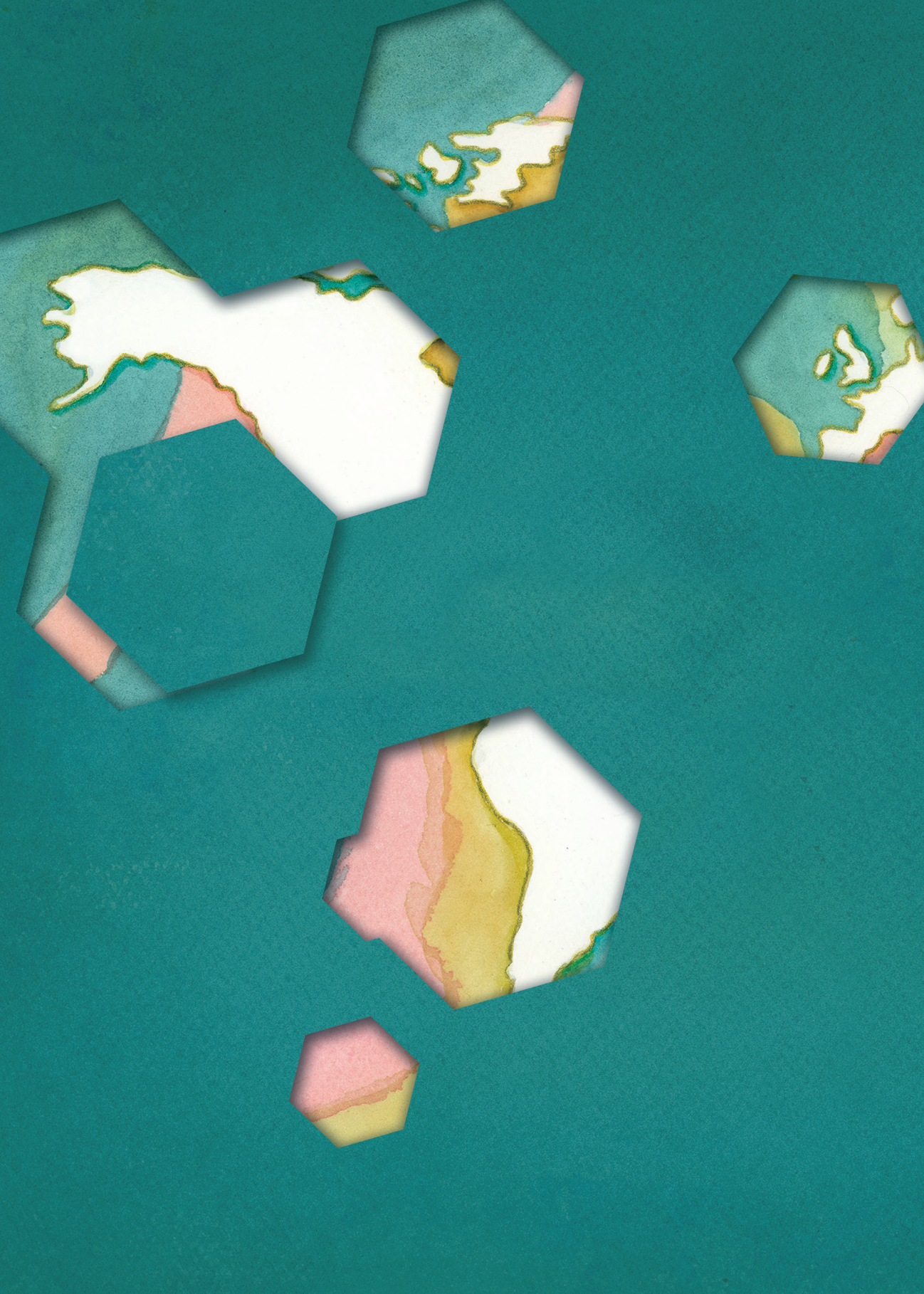
REFERENCES

1. da Silva EZ, Jamur MC, and Oliver C. Mast cell function: a new vision of an old cell. *J Histochem Cytochem* (2014) 62(10): 698-738.
2. Moon TC, St Laurent CD, Morris KE, Marcet C, Yoshimura T, Sekar Y, *et al*. Advances in mast cell biology: new understanding of heterogeneity and function. *Mucosal Immunol* (2010) 3(2): 111-28.
3. Metcalfe DD, Peavy RD, and Gilfillan AM. Mechanisms of mast cell signaling in anaphylaxis. *J Allergy Clin Immunol* (2009) 124(4): 639-46; quiz 647-8.
4. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, and Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* (2005) 23: 749-86.
5. Galli SJ, Tsai M, and Piliponsky AM. The development of allergic inflammation. *Nature* (2008) 454(7203): 445-54.
6. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G, *et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
7. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR, *et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
8. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
9. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, *et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
10. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
11. Abbring S, Verheijden KAT, Diks MAP, Leusink-Muis A, Hols G, Baars T, *et al*. Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model. *Front Immunol* (2017) 8: 1045.
12. Abbring S, Ryan JT, Diks MAP, Hols G, Garssen J, and van Esch BCAM. Suppression of food allergic symptoms by raw cow's milk in mice is retained after skimming but abolished after heating the milk - A promising contribution of alkaline phosphatase. *Nutrients* (2019) 11(7): 1499.
13. Abbring S, Wolf J, Ayechu Muruzabal V, Diks MAP, Alashkar Alhamwe B, Alhamdan F, *et al*. Raw cow's milk reduces allergic symptoms in a murine model for food allergy - A potential role for epigenetic modifications. *Nutrients* (2019) 11(8): 1721.
14. Abbring S, Hols G, Garssen J, and van Esch BCAM. Raw cow's milk consumption and allergic diseases - The potential role of bioactive whey proteins. *Eur J Pharmacol* (2019) 843: 55-65.
15. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
16. Perdijk O, van Splunter M, Savelkoul HFJ, Brugman S, and van Neerven RJJ. Cow's milk and immune function in the respiratory tract: potential mechanisms. *Front Immunol* (2018) 9: 143.
17. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G, *et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-1706 e13.

18. Malbec O, Roget K, Schiffer C, Iannascoli B, Dumas AR, Arock M, *et al*. Peritoneal cell-derived mast cells: an in vitro model of mature serosal-type mouse mast cells. *J Immunol* (2007) 178(10): 6465-75.
19. Abbring S, Kusche D, Roos TC, Diks MAP, Hols G, Garssen J, *et al*. 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(7): 1013-25.
20. Gurish MF and Austen KF. Developmental origin and functional specialization of mast cell subsets. *Immunity* (2012) 37(1): 25-33.
21. Dedkova EN, Sigova AA, and Zinchenko VP. Mechanism of action of calcium ionophores on intact cells: ionophore-resistant cells. *Membr Cell Biol* (2000) 13(3): 357-68.
22. Fattakhova G, Masilamani M, Borrego F, Gilfillan AM, Metcalfe DD, and Coligan JE. The high-affinity immunoglobulin-E receptor (FcepsilonRI) is endocytosed by an AP-2/clathrin-independent, dynamin-dependent mechanism. *Traffic* (2006) 7(6): 673-85.
23. Molfetta R, Gasparrini F, Peruzzi G, Vian L, Piccoli M, Frati L, *et al*. Lipid raft-dependent FcepsilonRI ubiquitination regulates receptor endocytosis through the action of ubiquitin binding adaptors. *PLoS One* (2009) 4(5): e5604.
24. Schuijs MJ, Willart MA, Hammad H, and Lambrecht BN. Cytokine targets in airway inflammation. *Curr Opin Pharmacol* (2013) 13(3): 351-61.
25. McLeod JJ, Baker B, and Ryan JJ. Mast cell production and response to IL-4 and IL-13. *Cytokine* (2015) 75(1): 57-61.
26. Simons K and Toomre D. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* (2000) 1(1): 31-9.
27. Baird B, Sheets ED, and Holowka D. How does the plasma membrane participate in cellular signaling by receptors for immunoglobulin E? *Biophys Chem* (1999) 82(2-3): 109-19.
28. Kovarova M, Tolar P, Arudchandran R, Draberova L, Rivera J, and Draber P. Structure-function analysis of Lyn kinase association with lipid rafts and initiation of early signaling events after Fcepsilon receptor I aggregation. *Mol Cell Biol* (2001) 21(24): 8318-28.
29. Field KA, Holowka D, and Baird B. Fc epsilon RI-mediated recruitment of p53/56lyn to detergent-resistant membrane domains accompanies cellular signaling. *Proc Natl Acad Sci USA* (1995) 92(20): 9201-5.
30. Frossi B, De Carli M, and Pucillo C. The mast cell: an antenna of the microenvironment that directs the immune response. *J Leukoc Biol* (2004) 75(4): 579-85.
31. Groschwitz KR and Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* (2009) 124(1): 3-20; quiz 21-2.



CHAPTER 10

General discussion

The potential of raw cow's milk to target allergic diseases is a hot topic that is discussed with passion. Headlines like 'raw milk: a superfood or super risky?' and 'the heated topic of raw milk' are regularly seen in the news. While advocates claim that raw milk has numerous health benefits that are eliminated by milk processing, skeptics argue that the scientific evidence is limited, that the nutritional value is not altered, and that its consumption is like playing Russian roulette because of the potential contamination with life-threatening pathogens. To stop this everlasting debate, the existing epidemiological evidence must be supported by a cause-effect relationship, and scientists and the dairy industry must join forces to develop microbiologically safe alternatives in the future. After all, the wide consumption of cow's milk makes it an attractive preventive strategy for allergic diseases if the risk of infections could be overcome.

This thesis aimed to investigate whether the current epidemiological evidence demonstrating an allergy-protective effect of raw cow's milk consumption can be strengthened by causality with the use of preclinical murine models. In addition, several research strategies were used to achieve a better understanding of the raw milk components involved in the allergy-protective effects and the underlying mechanisms. In this chapter, we summarize the main findings of this thesis and we discuss how the obtained results can promote the development of a safe and protective product in the future. Should we aim for 'rare' when the currently described associations between raw cow's milk consumption and the prevention of allergic diseases are confirmed? Should we stay with 'well done', because there is no safer option than heat treatment to prevent the risk of infections? Or is there a middle ground like 'medium'?

CONFIRMING THE ALLERGY-PROTECTIVE EFFECT

The remarkable increase in the prevalence of allergic diseases is particularly evident in Western countries and is therefore often explained by a reduced microbial exposure in early childhood as a consequence of urbanization (1). This so-called 'hygiene hypothesis' is supported by numerous large-scale epidemiological studies among European populations that have demonstrated that children growing up on a farm are at a significant lower risk of developing allergic diseases than children living in the same rural area but not on a farm (2-7). Farm-related exposures that contribute to this protective effect appeared to be contact with livestock (mostly cattle, pigs, and poultry), contact with animal feed (such as hay, grain, straw, and silage), and the consumption of raw, unprocessed, cow's milk (8-12). From these exposures, the consumption of raw cow's milk is particularly interesting, since its effect was the most robust and, moreover, independent of farm-related co-exposures and farming status (9-11). The observation is even more intriguing, given that milk is the most common food allergen in early childhood (13).

Even though there is mounting epidemiological evidence for the allergy-protective effect of raw cow's milk consumption (14, 15), the observed associations do not confirm a causal relationship. According to raw milk opponents, there is therefore a lack of substantiated evidence. They argue that the current studies do not contain any objective information about the raw milk status ([home] cooked or not) or a direct comparison with heat-treated milk (16, 17). Moreover, they believe that the protective effect is most likely related to the farming environment or to farm animals rather than to raw farm milk consumption (17). For ethical reasons, formal proof of the allergy-protective raw milk effect through controlled intervention studies in infants is not possible, but preclinical models offer an intermediate solution.

Most of the epidemiological studies have demonstrated a protective effect of raw cow's milk consumption on asthma (8, 9, 11, 14, 15). A well-defined murine house dust mite (HDM)-induced allergic asthma model was therefore used to examine whether these observed associations could be strengthened by causality. In line with the existing epidemiological studies, the results from **Chapter 3** confirm that raw cow's milk confers protection against allergic asthma. Strong protective effects on airway hyperresponsiveness and airway inflammation were observed which coincided with a reduced type 2 immune response. In addition to asthma, raw cow's milk also prevented the development of food allergy (as demonstrated in **Chapter 4**). Mice orally treated for eight consecutive days with raw milk before being sensitized and challenged with ovalbumin (OVA) showed reduced acute allergic symptoms upon challenge. This food allergy-protective effect was demonstrated in four different studies (**Chapters 4-7**), using four different raw milk batches, illustrating a consistent efficacy.

The fact that raw milk was able to prevent the development of HDM-induced allergic asthma and OVA-induced food allergy indicates that it can provide protection against an unrelated, non-milk, allergen. Since this protection was observed in the absence of the sensitizing allergen (i.e. HDM or OVA), raw milk exerts its allergy-protective effects via generic modulation of the immune system, reaching even beyond the gut. These results imply that raw cow's milk could be of great value to prevent sensitization against a wide range of allergens; from aeroallergens like HDM and pollen to food allergens like OVA and peanut. Moreover, they hint towards an even broader application, for example for the management of other immune-related disorders such as autoimmune diseases and type 2 diabetes.

In addition to demonstrating an allergy-protective effect of raw cow's milk consumption, the epidemiological evidence also showed a loss of protection upon milk processing. In contrast to raw milk, boiled raw milk and commercially available shop milk were not able to reduce allergy risk (14, 15). In **Chapter 3** and **Chapter 4** also these epidemiological findings were confirmed.

Heat-treated raw milk (80 °C, 10 min) showed no asthma-protective effect and processed, store-bought, milk (both pasteurized and homogenized) did not confer protection against food allergic symptoms. The obtained results thereby prove that although milk processing has no influence on the nutritional value of the milk, it is detrimental to the allergy-protective capacity.

RAW MILK COMPONENTS INVOLVED

Cow's milk produced or purchased directly on a farm differs in many respects from commercially available shop milk, even when the raw milk is skimmed or heated before consumption (18). Raw cow's milk does not undergo the processing steps necessary to preserve commercial milk along the supply chain. These processing steps usually consist of machine milking, milk fat standardization, homogenization, heat treatment, packaging, and storage (19, 20). Each of these steps induces profound changes in the milk composition and might explain why the beneficial raw milk effect is destroyed upon milk processing.

Comparing the allergy-protective capacity of raw cow's milk with commercially available shop milk (consumed by most people) as done in **Chapter 4**, was a logical first step to confirm the current epidemiological evidence. However, as these milk types differed in many aspects, amongst others in milk source, fat content, and heat treatment, it hardly provides insight in the raw milk components involved. In **Chapter 5** we therefore compared milk from the same origin differing in only one processing step.

Fat content and heat-sensitive components

Raw cow's milk contains numerous components with immunomodulatory properties such as immunoglobulins, lactoferrin, oligosaccharides, *n*-3 polyunsaturated fatty acids (PUFAs), vitamins, antioxidants, and anti-inflammatory cytokines (21). These bioactive components could theoretically all be responsible for the allergy-protective effects associated with raw cow's milk consumption (18, 21). However, since milk processing predominantly affects the fat content of the milk (by milk fat standardization and homogenization) and the heat-sensitive milk components (by heat treatment), the influence of these constituents is the most likely and therefore also the most studied. For both constituents, associations exist with regard to the asthma- and allergy-protective effects (14, 15, 22), but confirming that they are indeed responsible for the observed allergy protection by showing causality is crucial.

In **Chapter 5** we therefore investigated to which extent the fat content and the heat-sensitive components contribute to the allergy-protective effects of raw cow's milk by examining skimmed raw milk and pasteurized milk, respectively, in the previously used murine OVA-induced food allergy model. We showed that the suppression of food allergic symptoms by

raw cow's milk is retained after skimming but abolished after pasteurization of the milk. The results therefore indicate that not the fat content, but the heat-sensitive milk components are underlying the allergy-protective effects. Moreover, they emphasize that heating temperatures as low as those used during pasteurization (70-80 °C for 15-20 s (23)) are already detrimental to the allergy-protective effect. These results also imply that the loss of protection observed with commercial milk (**Chapter 4**) is not the result of homogenization, as previously suggested (24).

The observation that the fat content of the milk does not contribute to a large extent to the allergy-protective effects of raw cow's milk is in contrast with several epidemiological studies. According to both Waser *et al.* and Wijga *et al.*, frequent consumption of milk fat-containing products, such as full cream milk and butter is associated with a reduced asthma risk (9, 22). Brick *et al.* furthermore concluded that part of the asthma-protective effect of raw cow's milk is explained by a higher fat content and, particularly, higher *n*-3 PUFA levels compared to industrially processed milk (15). On the one hand these results stress the importance of demonstrating a cause-effect relationship, on the other hand the discrepancy could also be caused by the fact that the epidemiological studies focused on asthma whereas we focused on food allergy. In the HDM-induced allergic asthma model, as described in **Chapter 3** of this thesis, we observed a slight reduction in airway hyperresponsiveness when mice were treated with heated raw milk. The fat content of the milk is unaffected by heat and might therefore be responsible for this effect. Since we did not test the raw milk fat itself or a skimmed raw milk, we can, at this stage, only speculate that the fat content of raw milk does contribute to the observed asthma-protective effect. The raw milk components involved in the protective effect may therefore be dependent on the type of allergy.

Whey proteins

The importance of heat-sensitive components for the allergy-protective effect of raw cow's milk seems to be evident. Where epidemiological research already showed an association between heat treatment and loss of protection (14, 15), the results of **Chapter 3** and **Chapter 5** confirmed these findings. As source of many bioactive proteins, the heat-sensitive whey protein fraction is often related to the protective effects (21, 25). In addition to the major whey proteins β-lactoglobulin, α-lactalbumin, and bovine serum albumin (BSA), this fraction contains several less abundant proteins with immunomodulatory functionalities, including immunoglobulins, lactoferrin, enzymes, and cytokines (26). Heating can alter the physicochemical properties of these proteins and influence their biological impact (as reviewed in **Chapter 2**).

The first study to report a potential protective effect of the whey protein fraction of raw milk was the large epidemiological GABRIELA study conducted by Loss *et al.* among rural farm children (14). While the total protein content was not significantly related to asthma or atopy, the whey proteins β-lactoglobulin, α-lactalbumin, and BSA showed an inverse association with

asthma risk. Strikingly, these major whey proteins do not have immune-related functionalities that can directly be linked to the protective effects of raw cow's milk. It therefore remained to be elucidated whether the major whey proteins themselves confer the allergy-protective effect or whether they are a reflection of heat-sensitive whey proteins in general.

To achieve a better understanding of the mechanistic relation between heat damage to whey proteins and allergy development, native proteomics was combined with a functional readout for allergic diseases (**Chapter 6**). As expected, the number of native whey proteins decreased with increasing heat load. Based on the performed cluster analysis, the native whey protein profile significantly differed between milk heated ≥ 75 °C and milk heated < 75 °C. Comparable results were reported by Brick *et al.* who demonstrated a decrease in detectable native whey proteins after heating with an intensity higher than pasteurization (27). Where β-lactoglobulin, α-lactalbumin, and BSA all denatured at temperatures above 75 °C, a more in-depth analysis solely focusing on whey proteins with immune-related functionalities revealed that these proteins already started to denature from 65 °C. This is in line with previous studies, which demonstrated that particularly the immune active proteins in the whey fraction have a high heat sensitivity (28).

Interestingly, the loss of immunologically active whey proteins coincided with the loss of allergy protection observed in the murine OVA-induced food allergy model. The allergy-protective effect as observed when mice were treated with raw cow's milk lasted up to a heating temperature of 60 °C. Milk heated for 30 min above this temperature (65-80 °C) was no longer protective. Besides demonstrating the importance of immunologically active whey proteins that denature around 65 °C, these results also exclude the hypothesized contribution of β-lactoglobulin, α-lactalbumin, and BSA which all only denatured at temperatures above 75 °C. A closer look at the individual bioactive whey proteins showed a significant decrease in the concentration of complement C7, monocyte differentiation antigen CD14, and polymeric immunoglobulin receptor at 65 °C. The significant decrease in these proteins may (partly) explain the loss of protection at the same temperature. However, many other immunologically active whey proteins (e.g. immunoglobulins, lactoferrin) also showed a gradual decrease around 65 °C. Due to the large amount of immunologically active whey proteins present in raw cow's milk, even small changes in each of them can affect the final allergic response. In the end, the synergistic effect of changes in several of them simultaneously probably underlies the loss of protection.

The results described in this thesis demonstrate that even mild heating (i.e. 15 s at 78 °C as described in **Chapter 4** and 30 min at 65 °C as described in **Chapter 6**) is detrimental to the allergy-protective raw milk effect. These 'low' heating temperatures are generally not considered to be harmful to whey proteins because they are believed to denature at

temperatures greater than 75 °C (20, 29). Accordingly, we (**Chapter 6**) and others (27) showed that these mildly heated milk types have a similar overall native protein profile as raw milk. However, by omitting the major whey proteins, which indeed denatured above 75 °C, and specifically focusing on less abundant whey proteins with immunomodulatory properties, we observed that their denaturation already started at 65 °C. Much of the research investigating the effect of milk processing on whey proteins is performed with isolated ingredients. These studies have demonstrated that whey proteins such as immunoglobulins (30, 31) and lactoferrin (32-34) are not affected by the temperatures used during pasteurization, which is in contrast to our findings. Interestingly, research using the whole milk/whey fraction does show a reduced amount of these proteins after pasteurization (28). This discrepancy might be caused by the concurrent presence of β-lactoglobulin. At temperatures of around 70 °C, a previously hidden free thiol group (-SH group) in β-lactoglobulin becomes exposed (35). This free thiol group reacts with other whey proteins causing irreversible aggregation reactions (26, 36). These heat-induced aggregation reactions are likely to occur with TGF-β2 since this molecule has a strong hydrophobic character, favoring polymerization and non-specific interaction with other proteins (37). In addition, β-lactoglobulin also readily forms aggregates with immunoglobulins and lactoferrin (26). Whether the concomitant presence of β-lactoglobulin accelerates the denaturation of other whey proteins, making the temperature used during pasteurization harmful to the allergy-protective raw milk effect, is an interesting thought that requires further investigation. Moreover, this β-lactoglobulin hypothesis raises the question whether heat treatment might be less detrimental to human milk due to the absence of β-lactoglobulin.

Another approach to gain more insight into the raw milk components involved in the allergy-protective effect is to fractionate the milk based on molecular size. By investigating the capacity of these raw milk fractions to influence the allergic effector response *in vitro*, we have demonstrated that the heat-sensitive components contributing to the allergy-protective raw milk effect are likely to have a molecular weight of > 37 kDa (**Chapter 9**). Since β-lactoglobulin and α-lactalbumin have a molecular weight of < 25 kDa, this is another indication that these major whey proteins are not responsible for the observed protective effects. Characterization of the protective fractions by proteomics will give more insight into the actual raw milk protein(s) involved and might substantiate the findings of **Chapter 6**. After showing a direct impact on the allergic effector response, it would be interesting to assess whether raw milk fractions can also modulate epithelial, dendritic cell, and T cell responses, and, if so, whether the observed effects are caused by the same raw milk fractions or whether other components are involved.

Bacterial composition, microRNAs, and extracellular vesicles

Whereas this thesis focused primarily on the whey protein fraction in relation to the allergy-protective effects of raw cow's milk, we are aware that there are various other heat-sensitive raw milk constituents that may underlie the observed effects. The higher microbial load of raw

cow's milk is such an example (38). Raw cow's milk contains probiotic bacteria, described to have beneficial health effects (e.g. species of the genera *Lactobacillus* and *Bifidobacterium*), that could contribute to the observed protection (17, 39). However, their number is probably too low to have a physiological effect. In addition, total bacterial cell counts were not associated with asthma or atopy (14). Nevertheless, based on the current literature the contribution of bacteria cannot yet be excluded. Further investigation involving gamma-sterilized or filtered milk could contribute to elucidate the role of bacteria in the allergy-protective effect of raw cow's milk.

In addition, microRNAs are often mentioned as heat-sensitive, beneficial, raw milk ingredient. Specific microRNA species (mircoRNA-155, microRNA-148a, microRNA-29b, and microRNA-21) are hypothesized as potential contributors to the allergy-protective raw milk effect (40). However, these microRNAs were shown to be degraded after high heat treatment (as applied in boiled, ultra-high temperature [UHT] processing and extended shelf life [ESL] milk), but not after pasteurization (41). Since we observed a clear loss of allergy protection after pasteurization, the role of microRNAs is questionable.

Most of the milk-derived microRNAs are transported either in milk fat globules or in extracellular vesicles (EVs) (42). These EVs do not only carry microRNAs, but also lipids, functional proteins, and mRNA, and are recognized as potent vehicles for intercellular communication. Recently, both human and bovine milk EVs have been demonstrated to modulate immune cell function (43, 44). In a preliminary study, we therefore assessed the allergy-protective capacity of raw cow's milk-derived EVs. Treating mice for eight days with these EVs prior to OVA-sensitization significantly reduced the acute allergic skin response upon OVA challenge compared to PBS-treated control mice. This protective effect was not observed when mice were treated with EVs derived from processed milk. Since bovine milk EVs are known to be heat-sensitive (40), their contribution to the allergy-protective raw milk effect needs to be examined in more detail.

POTENTIAL UNDERLYING MECHANISMS

Besides the raw milk components involved, a better knowledge of the mechanisms underlying the allergy-protective effect of raw cow's milk will also enhance the development of microbiologically safe alternatives. The current literature mainly speculates on the formation of a tolerogenic environment which favors unresponsiveness upon allergen exposure (as reviewed in **Chapter 2**). The many immunomodulatory components present in raw cow's milk are hypothesized to promote regulatory T cell (Treg) development, to modulate the gut microbiome, and to enhance intestinal barrier function (21). However, none of these effects have actually been investigated after drinking raw milk.

Modulation of the T cell response

Raw milk exposure for eight days protected mice against OVA-induced allergic symptoms which indicates that these mice developed tolerance to OVA (**Chapter 5**). This immunological tolerance was established in the absence of the sensitizing allergen, demonstrating that raw cow's milk has the ability to induce tolerance via generic modulation of the immune system. Generic immunomodulation is based on the use of beneficial immunomodulatory components that can create the right environment for tolerance induction, and has already been described for several dietary components, such as prebiotics, probiotics, synbiotics, and *n*-3 PUFAs (45, 46).

Treg cells are identified as key players in inducing and maintaining immunological tolerance to allergens (47). They are believed to block the initiation of the allergic response through the suppression of antigen-presenting cells and allergen-specific Th2 cells. In addition, they shift immunoglobulin class switching in B cells from IgE to IgG4 and IgA, and they inhibit mast cell, basophil, and eosinophil activity. These inhibitory effects of Treg cells occur via the production of suppressive cytokines (IL-10 and TGF-β) and via cell-cell contact dependent mechanisms (48, 49). Even though raw cow's milk consumption was previously shown to be associated with increased FoxP3$^+$ Treg cell numbers in children (50), we could not fully confirm these findings. The percentage of CD25$^+$FoxP3$^+$ Treg cells in the spleen (**Chapter 6**) and the mesenteric lymph nodes (MLN; **Chapter 5**) was not affected by raw milk treatment. However, raw milk did increase the percentage of the Treg subtypes secreting TGF-β (Th3 cells; **Chapter 5**) and IL-10 (Tr1 cells; **Chapter 6**).

Induction of FoxP3$^+$ Treg cells occurs in the MLN via CD103$^+$ DCs. After acquiring antigen, these CD103$^+$ DCs migrate from the intestinal lamina propria to the MLN where they polarize naïve T cells into gut-homing FoxP3$^+$ Treg cells via a TGF-β- and retinoic acid-dependent mechanism (51, 52). While raw milk exposure did not significantly affect CD25$^+$FoxP3$^+$ Treg cells in the MLN, it did increase the tolerogenic CD103$^+$CD11b$^+$ DC subpopulation (**Chapter 5**). The fact that CD103$^+$ DCs induce the expression of gut-homing receptors on the cell surface of FoxP3$^+$ Treg cells suggests that the Treg cells might have migrated to the gut (52), but also in the intestinal lamina propria FoxP3$^+$ Treg cell frequency was not affected by raw milk. The late phase at which Treg cells were measured (at the end of the study, after allergy induction) might, however, not reflect the situation during the early tolerance induction phase where mice were treated with raw milk for eight consecutive days.

Even though we did not measure FoxP3$^+$ Treg numbers directly after raw milk exposure, we did look at histone acetylation of the FoxP3 gene at this timepoint (**Chapter 4**). Environmental factors such as microbes, stress, and tobacco smoke are known to interact with genes involved in allergy development through epigenetic mechanisms and we therefore assessed

whether this also applied to raw milk (53, 54). Epigenetic mechanisms affect the accessibility of the DNA to transcription enzymes without altering the DNA nucleotide sequence, which means that they can modulate the phenotype without affecting the genotype (53). In this way, epigenetic mechanisms are key in the plasticity of gene expression and are essential, for example, to the flexibility among $CD4^+$ T cell subsets (55). Interestingly, raw milk exposure for eight days increased histone acetylation of the FoxP3 gene compared to processed milk exposure in splenocyte-derived $CD4^+$ T cells. These results are in line with epidemiological studies which have shown that raw cow's milk consumption is associated with increased DNA demethylation of the FoxP3 gene (50). Furthermore, it has been demonstrated that acquiring tolerance in food allergic children involves epigenetic regulation of the FoxP3 gene (56). In summary, the increased $CD103^+$ DC numbers, increased Th3 and Tr1 cells, and increased histone acetylation of the FoxP3 gene after raw milk exposure provide a first indication that the induction of Treg cells possibly underlies the allergy-protective raw milk effect. However, formal proof, for example, via functionality assays or an adoptive transfer experiment in which raw milk-induced Treg cells are transferred to naïve recipient mice prior to allergen challenge should give a definite answer.

Besides increasing histone acetylation of the FoxP3 gene, raw milk also increased the acetylation of other T cell-related genes, such as IL-5 and T-bet. These results suggest that raw milk exposure for eight days induces a kind of general immune stimulation. After the induction of allergic symptoms, this general immune stimulation was resolved and raw milk specifically lowered histone acetylation of Th2-related genes (GATA3, IL-4, IL-5, and IL-13) of splenocyte-derived $CD4^+$ T cells. The reduced histone acetylation of Th2-related genes after allergy induction is in accordance with the protective effects observed on acute allergic symptoms and IgE levels and was furthermore reflected in a lower IL-5 (**Chapter 4**) and IL-13 (**Chapter 5**) production by *ex vivo* OVA-stimulated splenocytes. By targeting histone marks on T cell-related genes, raw milk might have induced immunological tolerance to OVA. Future research should assess whether this is a cause-effect relationship, for example by using histone acetyltransferase inhibitors, and whether effects are more pronounced with longer raw milk exposure.

Modulation of the T cell response was also observed in the HDM-induced allergic asthma model (**Chapter 3**). Raw milk suppressed the Th2-polarizing chemokine CCL17 in lung homogenates, reduced lung Th2 and Th17 cell frequency and inhibited IL-4 and IL-13 production after *ex vivo* restimulation of lung T cells with HDM. However, these effects were also observed in mice treated with heated raw milk, whereas these mice were not protected against asthma symptoms (airway hyperresponsiveness and airway inflammation), suggesting the involvement of an additional mechanism.

Inhibition of the allergic effector response

Mast cells are crucial effector cells in IgE-mediated allergic diseases, due to the high-affinity IgE receptor expressed on their cell membrane (57). A potential role of mast cells in the allergy-protective effect of raw cow's milk was suggested by the results of **Chapter 3**, where suppression of local type 2 cytokine levels, produced by other immune cells than T cells, seemed to be crucial for the raw milk-induced prevention of allergic asthma. Since it is well known that these type 2 cytokines can also be produced by basophils and mast cells (58, 59), we hypothesized a direct inhibitory effect of raw cow's milk on these effector cells.

In accordance with the hypothesis raised in **Chapter 3**, we demonstrated that raw cow's milk has the capacity to inhibit the allergic effector response *in vitro* by directly affecting mast cell activation (**Chapter 9**). Exposing mast cells to raw milk prior to activation reduced β-hexosaminidase release and *de novo* IL-6 and IL-13 production. We also showed that this reduction is likely caused by a reduced translocation of engaged FcεRI complexes into lipid rafts, crucial for FcεRI-mediated mast cell responses. The current findings thereby demonstrate that in addition to modulation of the T cell response, raw cow's milk is also able to directly influence the allergic effector response.

WHAT ABOUT ALLERGENICITY?

This thesis mainly focused on the tolerogenic feature of raw cow's milk and showed its potential to prevent both asthma and food allergy. This protective effect was clearly destroyed by milk processing; most likely because of a loss of functionality of immunomodulatory proteins present in the whey fraction of the milk. The findings of **Chapter 8** showed that milk processing also influences the context of allergen presentation to the immune system and thereby negatively affects the allergenic potential of the milk.

The effect of processing on the allergenicity of food proteins has been extensively studied and is highly dependent on the type of protein. High heat treatment was found to reduce the allergenicity of many food proteins, with birch tree pollen allergen Bet v 1 cross-reactive proteins in apple (Mal d 1) and carrot (Dau c) as classical examples. These proteins cause immediate oral symptoms in the uncooked form but are readily tolerated after heating, presumably by the loss of conformational epitopes (60, 61). However, in the case of peanut proteins, high temperatures may increase the allergenicity due to the Maillard reaction (glycation) that induces the aggregation of Ara h 2 proteins. These Ara h 2 aggregates were found to be more resistant to gastric digestion and have an enhanced IgE-binding capacity compared to unheated Ara h 2 (62, 63). With regard to milk proteins,

extensively heat-treated (baked) milk showed a reduced allergenicity in human trials (64-66), but the effect of lower heating temperatures, used during industrial milk processing, are less clear (67, 68).

Using isolated milk proteins, Roth-Walter *et al.* demonstrated that pasteurization-induced aggregation of α-lactalbumin and β-lactoglobulin resulted in enhanced allergic sensitization, as illustrated by increased IgE and Th2 cytokine responses (69). Using whole milk, we confirmed this increase in sensitizing capacity upon heat treatment (**Chapter 8**). We furthermore showed that the reduced sensitizing capacity of raw milk is retained in the isolated native whey protein fraction of the milk, indicating that the effect is independent of the caseins. Since the allergenicity of milk is not only determined by its sensitizing capacity (i.e. production of allergen-specific IgE antibodies), but also by its capacity to bind to IgE antibodies and thereby induce an allergic reaction (23), we assessed whether native whey proteins also have a lower capacity to elicit an allergic response when sensitization to heated whey proteins already occurred. Interestingly, this indeed appeared to be the case, hinting towards a potential therapeutic applicability.

The reduced allergenicity of raw cow's milk was confirmed in a proof-of-concept provocation pilot, in which cow's milk allergic children tolerated raw cow's milk up to the maximum level of 50 mL, whereas, in most cases, the provocation with commercially available shop milk had to be stopped earlier due to the development of allergic symptoms. Overall, these findings demonstrate that, in addition to the use of extremely high temperatures, a reduced allergenicity can also be achieved by keeping the milk proteins in their native state. We therefore hypothesize that the allergenicity of cow's milk is following a parabolic shape; with a low allergenic potential at low (< 50 °C, e.g. raw cow's milk) and extremely high temperatures (> 180 °C, e.g. baked milk), and an increasing allergenic potential with temperatures in between.

MICROBIOLOGICALLY SAFE ALTERNATIVES

When produced under strict hygienic and microbiological standards, the risks of consuming raw cow's milk are rather low (70). Nevertheless, a zero-risk can never be attained, and the consumption of raw cow's milk is therefore strongly discouraged or even prohibited by regulatory authorities (71). The current literature mainly speculates about mildly processed milk, in which bioactive raw milk components are retained, and about specific raw milk ingredients for supplementing processed milk, as potential, microbiologically safe, alternatives to raw cow's milk (18, 21, 27, 72). However, scientific evidence to substantiate these hypotheses is lacking.

In **Chapter 5** and **Chapter 7** of this thesis we showed that spiking processed milk with bioactive whey proteins is a promising preventive approach for allergic diseases as alternative to raw cow's milk. As a first proof-of-concept, we spiked pasteurized milk with alkaline phosphatase. Alkaline phosphatase is used in dairy industry to verify successful pasteurization (73) and is one of the first bioactive whey proteins losing its activity upon heat treatment, making it a likely allergy-protective candidate. A 10× higher alkaline phosphatase concentration than present in raw cow's milk was able to fully restore the allergy-protective effect in the food allergy model used (**Chapter 5**). A 5× higher concentration was also promising, although less effective (**Chapter 7**). In 5× higher concentrations, the bioactive whey proteins lactoferrin, osteopontin, and IgG showed promising effects as well, but it turned out to be the combination of the four (including alkaline phosphatase) that most closely resembled the allergy-protective raw milk effect (**Chapter 7**). Interactions between these whey proteins have been described, osteopontin, for example, protects lactoferrin from proteolysis upon ingestion (74), and may underlie the allergy-protective raw milk effect, emphasizing the importance of the milk matrix.

By developing finer processing methods to selectively remove pathogens while retaining the beneficial raw milk effects, the raw milk matrix can be maintained. Filtration is such a method and was applied to obtain the native whey protein fraction as used in **Chapter 8** of this thesis. These native whey proteins showed a reduced allergenicity compared to their processed counterparts. Their tolerogenic capacity remains to be assessed, but since whey proteins are the basis of many infant formulas, native whey proteins can be an interesting raw milk alternative with great potential.

FUTURE PERSPECTIVES

The capacity of raw cow's milk to protect against unrelated, non-milk, allergens through generic modulation of the immune system, reaching even beyond the gut, offers a wide range of future possibilities. In addition to allergic diseases, the findings of this thesis imply that raw milk may also confer protection against other immune-related disorders or may even act as adjuvant to improve vaccine-specific immune responses or to increase allergen-specific immunotherapy efficacy. Moreover, the better tolerability of raw cow's milk in cow's milk allergic children was accompanied by a reduction in other allergic symptoms, hinting towards a potential therapeutic applicability.

Besides investigating the broad applicability of raw cow's milk, future studies should assess the role of the gut microbiome. Raw milk components such as lactoferrin have been demonstrated to promote the growth *Bifidobacteria* and *Lactobacilli* (75, 76) and similar effects have been hypothesized for lactose and milk oligosaccharides (21). *Bifidobacteria*

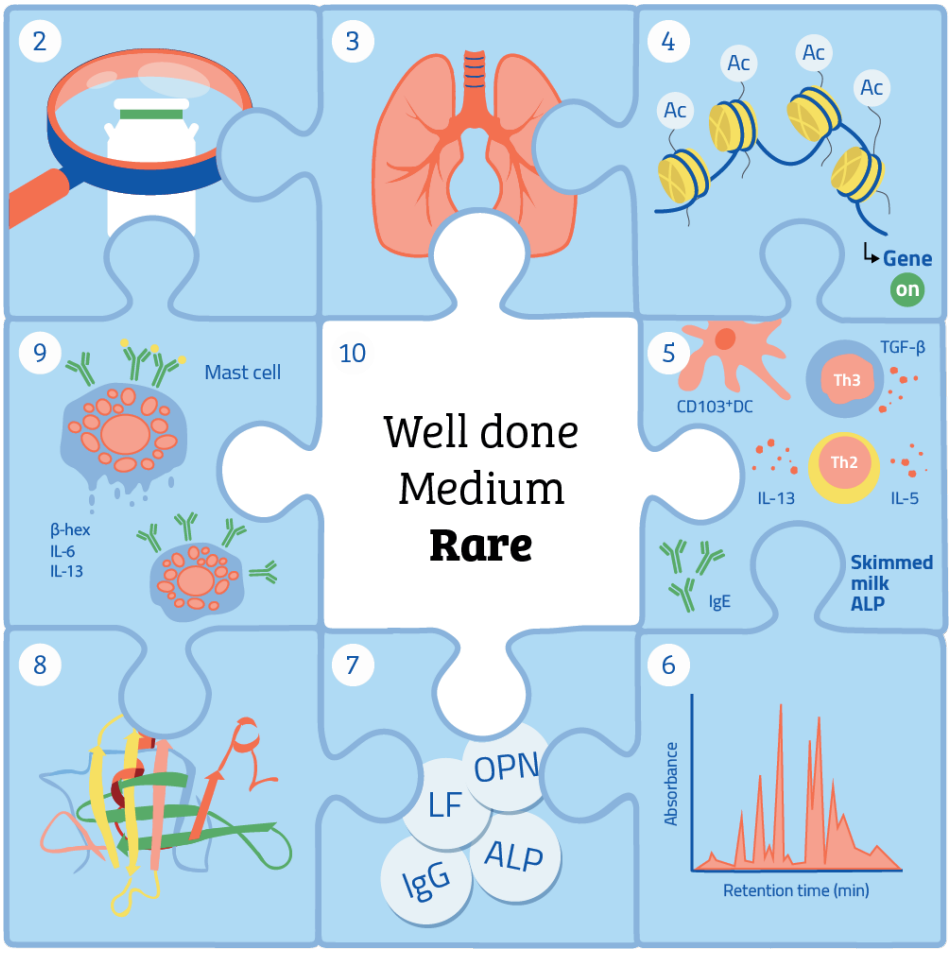
and *Lactobacilli* are considered to be beneficial to the host and their presence in the gut microbiota of infants correlates with protection against allergic diseases (77, 78). In addition, these bacteria are potent producers of short-chain fatty acids (SCFA) by fermenting non-digestible oligosaccharides in the colon. These SCFA (butyrate, acetate, and propionate) have the capacity to prevent the development of allergic diseases by enhancing epithelial integrity, inhibiting mast cell activation, and promoting Treg cell differentiation and IgA release from plasma cells (79-81). In our experiments, raw milk did not affect caecal SCFA concentrations (**Chapter 5**), but effects on the gut microbiota itself were not assessed and the timing of measuring these SCFA levels might be crucial (directly after raw milk exposure instead of after the induction of allergic symptoms).

Although many other future directions can be devised, a change of mindset is in my opinion the most important. The allergy-protective effect of raw cow's milk can no longer be neglected, and the dairy industry should react accordingly. We demonstrated the potential of finer processing methods, for example to obtain a native whey fraction that can be used in whey-based infant formulas. We also showed that bioactive whey proteins can be added to processed milk to mimic the allergy-protective raw milk effect. And although I believe that we should aim at a cleaner milk production, heating raw milk at a maximum temperature of 60 °C is perhaps the easiest alternative. In the future, we should therefore define whether raw milk heated for 30 min at 60 °C is microbiologically safe. In addition, it would be interesting to examine whether batch pasteurization (30 min at 62.5 °C), commonly applied to human donor milk (82), is detrimental to the allergy-protective effects, since the temperature used is on the border of allergy protection (60 vs 65 °C).

OVERALL CONCLUSION – 'WELL DONE, MEDIUM OR RARE?'

This thesis provides scientifically substantiated evidence that confirms the allergy-protective effect of raw cow's milk consumption, as previously demonstrated by epidemiological studies. For the first time, a causal relationship could be demonstrated by using preclinical murine models. Raw cow's milk prevented both asthma and food allergy and this protective effect was clearly destroyed by milk processing. The findings of this thesis furthermore show that heat-sensitive whey proteins that denature around 65 °C are most likely responsible for the allergy-protective raw milk effect and that both modulation of the T cell response and inhibition of the allergic effector response are underlying the protective effects. Besides a tolerogenic feature, raw cow's milk and native whey proteins also showed to have a lower allergenicity compared to their processed counterparts.

To conclude, it may be clear that the current industrial milk processing techniques (i.e. standardization, homogenization, and heat treatment) are detrimental to the allergy-protective effects of raw cow's milk; staying with 'well done' is therefore not an option. Solely heating raw milk, as middle ground ('medium'), is also no option, when temperatures above 60 °C are used. Spiking processed milk with bioactive whey proteins, on the contrary, can also be considered as 'medium' and was proven to be a promising raw milk alternative. However, the fact that the currently described associations between raw cow's milk consumption and allergic diseases are confirmed by causality clearly advocates for 'rare', and the strength and robustness of the findings emphasize that we should aim at a cleaner milk production. Since the potential risk of infections will always be a counterargument, 'rare' should also be considered as heating raw milk to a maximum temperature of 60 °C or as using milder processing methods, as long as the bioactive whey proteins are retained (**Summarizing Figure 1**).



◂ **Summarizing Figure 1.** The search for an answer to the question; 'well done, medium or rare?' started with an extensive literature search on the role of raw cow's milk in allergic diseases (**2**). By using preclinical murine models, the currently described associations between raw cow's milk consumption and the prevention of asthma could be confirmed (**3**). Raw cow's milk was furthermore able to prevent food allergy, potentially by altering epigenetic marks on T cell-related genes (**4**). The allergy-protective effect of raw cow's milk was found to be retained after skimming but abolished after pasteurization of the milk, indicating that not the fat content, but heat-sensitive milk components are underlying the protective effects. The protection by raw and skimmed raw cow's milk was furthermore accompanied by an induction of tolerance-associated cell types, demonstrating modulation of the T cell response (**5**). The loss of allergy protection at heating temperatures of 65 °C and above coincided with a loss of whey proteins with immune-related functionalities (**6**). Spiking processed milk with such bioactive whey proteins turned out to be an interesting raw milk alternative (**7**). Besides a tolerogenic feature, raw cow's milk and native whey proteins showed to have a lower allergenicity compared to their processed counterparts (**8**). Next to the described capacity to modulate T cell responses, raw cow's milk was also able to inhibit the allergic effector response (**9**). Together, the findings in this thesis clearly advocate for 'rare' (**10**). The epidemiological evidence is confirmed by causality, so the potential of raw cow's milk to prevent allergic diseases can no longer be neglected. Aiming at a cleaner milk production is therefore essential. However, since the risk of infections will always exist, 'rare' should also be considered as heating raw milk to a maximum temperature of 60 °C or as using finer processing methods, as long as the bioactive whey proteins are retained. The currently used industrial milk processing methods, considered as 'well done', are detrimental to the allergy-protective raw milk effect and a middle ground ('medium') cannot be found in solely heating raw milk, when temperatures above 60 °C are applied. Spiking processed milk with bioactive whey proteins, on the contrary, can also be considered as 'medium' and showed great potential as raw milk alternative.

REFERENCES

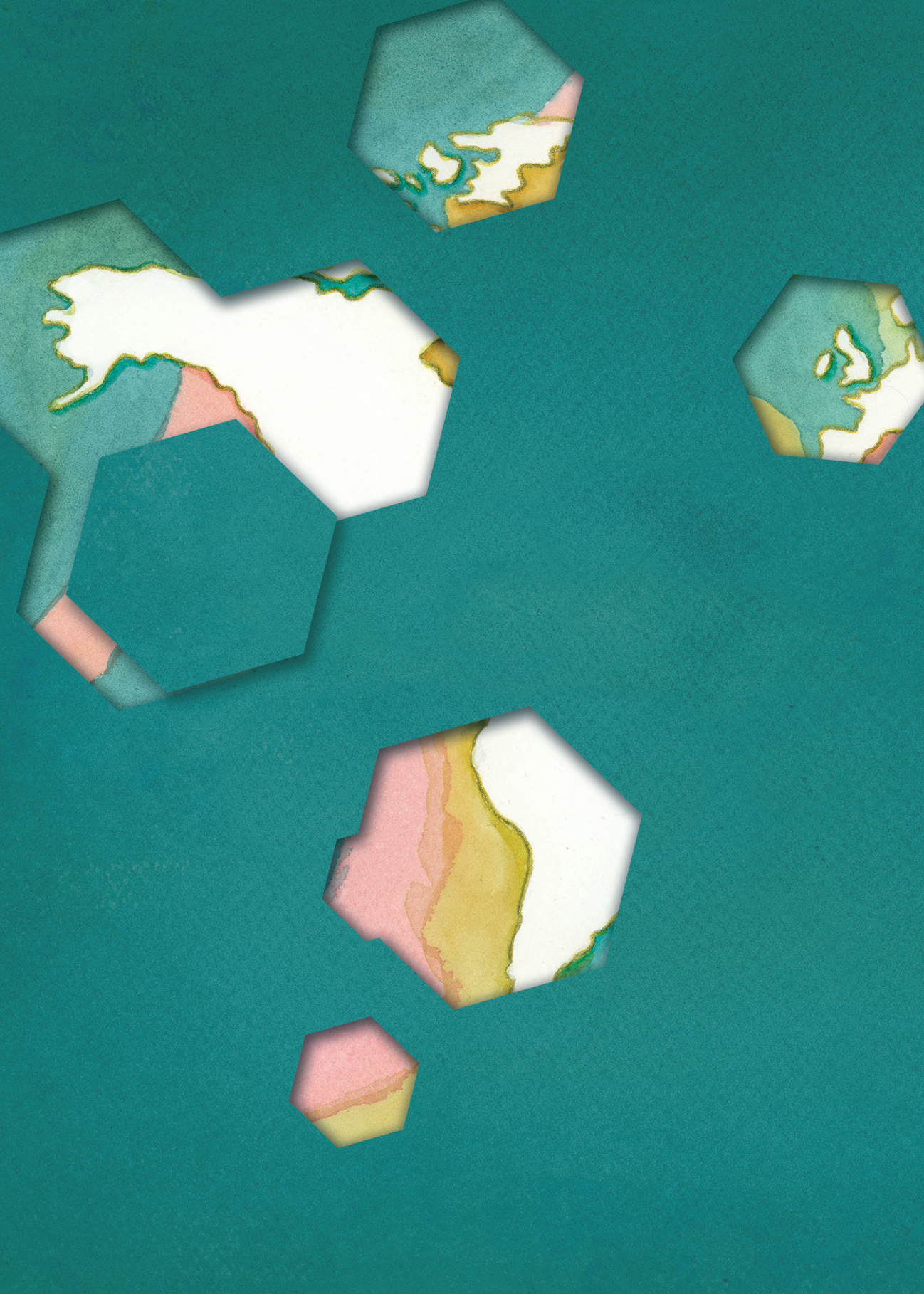
1. Strachan DP. Hay fever, hygiene, and household size. *Brit Med J* (1989) 299(6710): 1259-60.
2. Kilpelainen M, Terho EO, Helenius H, and Koskenvuo M. Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy* (2000) 30(2): 201-8.
3. Braun-Fahrlander C, Gassner M, Grize L, Neu U, Sennhauser FH, Varonier HS*, et al*. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss Study on Childhood Allergy and Respiratory Symptoms with Respect to Air Pollution. *Clin Exp Allergy* (1999) 29(1): 28-34.
4. von Ehrenstein OS, von Mutius E, Illi S, Baumann L, Bohm O, and von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* (2000) 30(2): 187-93.
5. Riedler J, Eder W, Oberfeld G, and Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin Exp Allergy* (2000) 30(2): 194-200.
6. Alfven T, Braun-Fahrlander C, Brunekreef B, von Mutius E, Riedler J, Scheynius A*, et al*. Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle - The PARSIFAL study. *Allergy* (2006) 61(4): 414-21.
7. Ernst P and Cormier Y. Relative scarcity of asthma and atopy among rural adolescents raised on a farm. *Am J Respir Crit Care Med* (2000) 161(5): 1563-6.
8. Ege MJ, Frei R, Bieli C, Schram-Bijkerk D, Waser M, Benz MR*, et al*. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol* (2007) 119(5): 1140-7.
9. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E*, et al*. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* (2007) 37(5): 661-70.
10. Perkin MR and Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? *J Allergy Clin Immunol* (2006) 117(6): 1374-81.
11. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S*, et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* (2001) 358(9288): 1129-33.
12. Remes ST, Iivanainen K, Koskela H, and Pekkanen J. Which factors explain the lower prevalence of atopy amongst farmers' children? *Clin Exp Allergy* (2003) 33(4): 427-34.
13. Skripak JM, Matsui EC, Mudd K, and Wood RA. The natural history of IgE-mediated cow's milk allergy. *J Allergy Clin Immunol* (2007) 120(5): 1172-7.
14. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G*, et al*. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* (2011) 128(4): 766-73 e4.
15. Brick T, Schober Y, Bocking C, Pekkanen J, Genuneit J, Loss G*, et al*. Omega-3 fatty acids contribute to the asthma-protective effect of unprocessed cow's milk. *J Allergy Clin Immunol* (2016) 137(6): 1699-1706 e13.
16. Lucey JA. Raw milk consumption: risks and benefits. *Nutr Today* (2015) 50(4): 189-93.
17. Claeys WL, Cardoen S, Daube G, De Block J, Dewettinck K, Dierick K*, et al*. Raw or heated cow milk consumption: review of risks and benefits. *Food Control* (2013) 31(1): 251-62.
18. Braun-Fahrländer C and von Mutius E. Can farm milk consumption prevent allergic diseases? *Clin Exp Allergy* (2011) 41(1): 29-35.

19. Michalski MC and Januel C. Does homogenization affect the human health properties of cow's milk? *Trends Food Sci Tech* (2006) 17(8): 423-37.
20. Chandan RC. Dairy ingredients for food processing: an overview in *Dairy Ingredients for Food Processing*. (2011): 3-33.
21. van Neerven RJ, Knol EF, Heck JM, and Savelkoul HF. Which factors in raw cow's milk contribute to protection against allergies? *J Allergy Clin Immunol* (2012) 130(4): 853-8.
22. Wijga AH, Smit HA, Kerkhof M, de Jongste JC, Gerritsen J, Neijens HJ*, et al*. Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: the PIAMA birth cohort study. *Thorax* (2003) 58(7): 567-72.
23. Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S*, et al*. Food processing and allergenicity. *Food Chem Toxicol* (2015) 80: 223-40.
24. Miller JD. Absence of homogenization might explain the benefits of raw cow's milk. *J Allergy Clin Immunol* (2013) 131(3): 927.
25. Sozańska B. Raw cow's milk and its protective effect on allergies and asthma. *Nutrients* (2019) 11(2): 469.
26. Chatterton DE, Nguyen DN, Bering SB, and Sangild PT. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int J Biochem Cell Biol* (2013) 45(8): 1730-47.
27. Brick T, Ege M, Boeren S, Bock A, von Mutius E, Vervoort J*, et al*. Effect of processing intensity on immunologically active bovine milk serum proteins. *Nutrients* (2017) 9(9): 963.
28. Zhang LN, Boeren S, Smits M, van Hooijdonk T, Vervoort J, and Hettinga K. Proteomic study on the stability of proteins in bovine, camel, and caprine milk sera after processing. *Food Res Int* (2016) 82: 104-11.
29. Laleye L, Jobe B, and Wasesa A. Comparative study on heat stability and functionality of camel and bovine milk whey proteins. *J Dairy Sci* (2008) 91(12): 4527-34.
30. Collins AM, Roberton DM, Hosking CS, and Flannery GR. Bovine milk, including pasteurised milk, contains antibodies directed against allergens of clinical importance to man. *Int Arch Allergy Appl Immunol* (1991) 96(4): 362-7.
31. Mainer G, Sanchez L, Ena JM, and Calvo M. Kinetic and thermodynamic parameters for heat denaturation of bovine milk IgG, IgA and IgM. *J Food Sci* (1997) 62(5): 1034-8.
32. Paulsson MA, Svensson U, Kishore AR, and Naidu AS. Thermal behavior of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. *J Dairy Sci* (1993) 76(12): 3711-20.
33. Oria R, Ismail M, Sanchez L, Calvo M, and Brock JH. Effect of heat treatment and other milk proteins on the interaction of lactoferrin with monocytes. *J Dairy Res* (1993) 60(3): 363-9.
34. Schwarcz WD, Carnelocce L, Silva JL, Oliveira AC, and Goncalves RB. Conformational changes in bovine lactoferrin induced by slow or fast temperature increases. *Biol Chem* (2008) 389(8): 1137-42.
35. Sava N, Van der Plancken I, Claeys W, and Hendrickx M. The kinetics of heat-induced structural changes of beta-lactoglobulin. *J Dairy Sci* (2005) 88(5): 1646-53.
36. Wijayanti HB, Bansal N, and Deeth HC. Stability of whey proteins during thermal processing: a review. *Compr Rev Food Sci F* (2014) 13(6): 1235-51.
37. Akbache A, Rocafi A, Saffon M, Lamiot E, Moroni O, Turgeon S*, et al*. Effect of heating on the distribution of transforming growth factor-beta 2 in bovine milk. *Food Res Int* (2011) 44(1): 28-32.
38. Perkin MR. Unpasteurized milk: health or hazard? *Clin Exp Allergy* (2007) 37(5): 627-30.
39. Ishibashi N and Yamazaki S. Probiotics and safety. *Am J Clin Nutr* (2001) 73(2 Suppl): 465S-70S.
40. Melnik BC, John SM, and Schmitz G. Milk: an exosomal microRNA transmitter promoting thymic regulatory T cell maturation preventing

the development of atopy? *J Transl Med* (2014) 12(1): 43.
41. Kirchner B, Pfaffl MW, Dumpler J, von Mutius E, and Ege MJ. MicroRNA in native and processed cow's milk and its implication for the farm milk effect on asthma. *J Allergy Clin Immunol* (2016) 137(6): 1893-5 e13.
42. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, *et al*. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. *J Dairy Sci* (2015) 98(5): 2920-33.
43. Admyre C, Johansson SM, Qazi KR, Filén J-J, Lahesmaa R, Norman M, *et al*. Exosomes with immune modulatory features are present in human breast milk. *J Immunol* (2007) 179(3): 1969-78.
44. Arntz OJ, Pieters BC, Oliveira MC, Broeren MG, Bennink MB, de Vries M, *et al*. Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models. *Mol Nutr Food Res* (2015) 59(9): 1701-12.
45. Vonk MM, Kostadinova AI, Kopp MV, van Esch BCAM, Willemsen LEM, Knippels LMJ, *et al*. 16 - Dietary interventions in infancy in *Allergy, Immunity and Tolerance in Early Childhood*, U. Wahn and H.A. Sampson, Editors. (2016); 261-84.
46. Gourbeyre P, Denery S, and Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. *J Leukoc Biol* (2011) 89(5): 685-95.
47. Soyer OU, Akdis M, Ring J, Behrendt H, Crameri R, Lauener R, *et al*. Mechanisms of peripheral tolerance to allergens. *Allergy* (2013) 68(2): 161-70.
48. Xystrakis E, Boswell SE, and Hawrylowicz CM. T regulatory cells and the control of allergic disease. *Expert Opin Biol Ther* (2006) 6(2): 121-33.
49. Akdis M, Blaser K, and Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* (2005) 116(5): 961-8; quiz 969.
50. Lluis A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D, *et al*. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol* (2014) 133(2): 551-9.
51. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, *et al*. A functionally specialized population of mucosal $CD103^+$ DCs induces $Foxp3^+$ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* (2007) 204(8): 1757-64.
52. Scott CL, Aumeunier AM, and Mowat AM. Intestinal $CD103^+$ dendritic cells: master regulators of tolerance? *Trends Immunol* (2011) 32(9): 412-9.
53. Potaczek DP, Harb H, Michel S, Alaskhar Alhamwe B, Renz H, and Tost J. Epigenetics and allergy: from basic mechanisms to clinical applications. *Epigenomics* (2017) 9(4): 539-71.
54. Alaskhar Alhamwe B, Khalaila R, Wolf J, von Bulow V, Harb H, Alhamdan F, *et al*. Histone modifications and their role in epigenetics of atopy and allergic diseases. *Allergy Asthma Clin Immunol* (2018) 14: 39.
55. Suarez-Alvarez B, Rodriguez RM, Fraga MF, and Lopez-Larrea C. DNA methylation: a promising landscape for immune system-related diseases. *Trends Genet* (2012) 28(10): 506-14.
56. Paparo L, Nocerino R, Cosenza L, Aitoro R, D'Argenio V, Del Monaco V, *et al*. Epigenetic features of FoxP3 in children with cow's milk allergy. *Clin Epigenetics* (2016) 8(1): 86.
57. Metcalfe DD, Peavy RD, and Gilfillan AM. Mechanisms of mast cell signaling in anaphylaxis. *J Allergy Clin Immunol* (2009) 124(4): 639-46; quiz 647-8.
58. Schuijs MJ, Willart MA, Hammad H, and Lambrecht BN. Cytokine targets in airway inflammation. *Curr Opin Pharmacol* (2013) 13(3): 351-61.
59. McLeod JJ, Baker B, and Ryan JJ. Mast cell production and response to IL-4 and IL-13. *Cytokine* (2015) 75(1): 57-61.

60. Vieths S, Scheurer S, and Ballmer-Weber B. Current understanding of cross-reactivity of food allergens and pollen. *Ann NY Acad Sci* (2002) 964(1): 47-68.
61. Nowak-Wegrzyn A and 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(3): 234-7.
62. Maleki SJ, Viquez O, Jacks T, Dodo H, Champagne ET, Chung SY*, et al*. The major peanut allergen, Ara h 2, functions as a trypsin inhibitor, and roasting enhances this function. *J Allergy Clin Immunol* (2003) 112(1): 190-5.
63. Gruber P, Becker W-M, and Hofmann T. Influence of the Maillard reaction on the allergenicity of rAra h 2, a recombinant major allergen from peanut (Arachis hypogaea), its major epitopes, and peanut agglutinin. *J Agr Food Chem* (2005) 53(6): 2289-96.
64. Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N*, et al*. Tolerance to extensively heated milk in children with cow's milk allergy. *J Allergy Clin Immunol* (2008) 122(2): 342-7, 347 e1-2.
65. Mehr S, Turner PJ, Joshi P, Wong M, and Campbell DE. Safety and clinical predictors of reacting to extensively heated cow's milk challenge in cow's milk-allergic children. *Ann Allergy Asthma Immunol* (2014) 113(4): 425-9.
66. Caubet JC, Nowak-Wegrzyn A, Moshier E, Godbold J, Wang J, and Sampson HA. Utility of casein-specific IgE levels in predicting reactivity to baked milk. *J Allergy Clin Immunol* (2013) 131(1): 222-4. e4.
67. Poulsen OM, Hau J, and Kollerup J. Effect of homogenization and pasteurization on the allergenicity of bovine milk analysed by a murine anaphylactic shock model. *Clin Allergy* (1987) 17(5): 449-58.
68. Host A and Samuelsson EG. Allergic reactions to raw, pasteurized, and homogenized/pasteurized cow milk: a comparison. A double-blind placebo-controlled study in milk allergic children. *Allergy* (1988) 43(2): 113-8.
69. Roth-Walter F, Berin MC, Arnaboldi P, Escalante CR, Dahan S, Rauch J*, et al*. Pasteurization of milk proteins promotes allergic sensitization by enhancing uptake through Peyer's patches. *Allergy* (2008) 63(7): 882-90.
70. Bundesministerium der Justiz und für Verbraucherschutz. Verordnung über die Güteprüfung und Bezahlung der Anlieferungsmilch (Milch-Güteverordnung). (1980) Available from: http://www.gesetze-im-internet.de/milchg_v/index.html Accessed on: 11 August 2019
71. Committee on Infectious Diseases & Committee on Nutrition & American Academy of Pediatrics. Consumption of raw or unpasteurized milk and milk products by pregnant women and children. *Pediatrics* (2014) 133(1): 175-9.
72. Yu JE and Miller RL. Got milk? Understanding the farm milk effect in allergy and asthma prevention. *J Allergy Clin Immunol* (2016) 137(6): 1707-8.
73. Rankin SA, Christiansen A, Lee W, Banavara DS, and Lopez-Hernandez A. Invited review: the application of alkaline phosphatase assays for the validation of milk product pasteurization. *J Dairy Sci* (2010) 93(12): 5538-51.
74. Yamniuk AP, Burling H, and Vogel HJ. Thermodynamic characterization of the interactions between the immunoregulatory proteins osteopontin and lactoferrin. *Mol Immunol* (2009) 46(11-12): 2395-402.
75. Giansanti F, Panella G, Leboffe L, and Antonini G. Lactoferrin from milk: nutraceutical and pharmacological properties. *Pharmaceuticals* (2016) 9(4): 61.
76. Oda H, Wakabayashi H, Yamauchi K, and Abe F. Lactoferrin and bifidobacteria. *Biometals* (2014) 27(5): 915-22.
77. Bjorksten B, Sepp E, Julge K, Voor T, and Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* (2001) 108(4): 516-20.

78. Sjogren YM, Jenmalm MC, Bottcher MF, Bjorksten B, and Sverremark-Ekstrom E. Altered early infant gut microbiota in children developing allergy up to 5 years of age. *Clin Exp Allergy* (2009) 39(4): 518-26.
79. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, and Macia L. Chapter three - The role of short-chain fatty acids in health and disease in *Advances in Immunology*, F.W. Alt, Editor. (2014); 91-119.
80. McKenzie C, Tan J, Macia L, and Mackay CR. The nutrition-gut microbiome-physiology axis and allergic diseases. *Immunol Rev* (2017) 278(1): 277-95.
81. Tan J, McKenzie C, Vuillermin PJ, Goverse G, Vinuesa CG, Mebius RE*, et al*. Dietary fiber and bacterial SCFA enhance oral tolerance and protect against food allergy through diverse cellular pathways. *Cell Rep* (2016) 15(12): 2809-24.
82. Peila C, Moro GE, Bertino E, Cavallarin L, Giribaldi M, Giuliani F*, et al*. The effect of Holder pasteurization on nutrients and biologically-active components in donor human milk: a review. *Nutrients* (2016) 8(8): 477.



APPENDICES

NEDERLANDSE SAMENVATTING

Het wel of niet drinken van rauwe, onbewerkte, koemelk is een veelbesproken onderwerp. Voorstanders beweren dat rauwe melk tal van gezondheidsvoordelen heeft die worden vernietigd tijdens het bewerkingsproces, terwijl tegenstanders het drinken van rauwe melk afraden vanwege het risico op voedselinfecties en het ontbreken van wetenschappelijk onderbouwd bewijs. De mogelijke aanwezigheid van ziekteverwekkers zoals *Campylobacter*, *Listeria*, *Salmonella* en *Escherichia coli* is reden voor overheidsinstanties om het drinken van rauwe melk sterk af te raden; zij adviseren om de melk te koken voor consumptie. Tegelijkertijd is er een groeiende groep consumenten die naar de boer gaat voor rauwe melk omdat ze de voorkeur geeft aan natuurlijke, onbewerkte, voedingsproducten.

Ondanks het risico op voedselinfecties wordt rauwe melk door een aanzienlijk aantal mensen gedronken. Met name melkveehouderijfamilies, maar tot op zekere hoogte ook landelijke, niet-agrarische families, geven er vaak de voorkeur aan om de melk onbewerkt te drinken. Deze families maakten het mogelijk om de potentiële gezondheidsbevorderende effecten van rauwe melk nader te onderzoeken.

In de afgelopen decennia nam het aantal mensen dat lijdt aan een allergie sterk toe. Aangezien deze toename vooral zichtbaar is in welvarende, Westerse landen, wordt hij vaak toegeschreven aan de verstedelijking en de daarmee gepaard gaande verbetering in hygiëne. Bijbehorende veranderingen in voeding en levensstijl, zoals kleinere gezinnen, betere sanitaire voorzieningen en een hoger antibiotica gebruik leiden tot een verminderde blootstelling aan bacteriën op jonge leeftijd. Hierdoor leert het ontwikkelende afweersysteem niet wanneer het wel en wanneer het niet hoeft te reageren op externe of interne prikkels. Dit wordt ook wel de hygiënehypothese genoemd en in overeenstemming met deze hypothese is er door talloze grootschalige epidemiologische onderzoeken aangetoond dat kinderen die opgroeien op een boerderij minder kans hebben op het ontwikkelen van allergieën dan kinderen die opgroeien in dezelfde omgeving maar niet op een boerderij. Factoren die bijdragen aan dit beschermende effect zijn blootstelling aan vee en veevoer en het drinken van rauwe, onbewerkte koemelk. Met name het beschermende effect van rauwe koemelk is interessant omdat dit effect onafhankelijk bleek te zijn van andere, gelijktijdige, blootstellingen maar ook van het wel of niet opgroeien op een boerderij. Dit suggereert dat iedereen baat zou kunnen hebben bij het drinken van rauwe koemelk.

Het allergie-beschermende effect lijkt echter beperkt te zijn tot het drinken van rauwe koemelk. Industrieel bewerkte melk was namelijk niet in staat om het risico op allergieën te verminderen, wat suggereert dat melkverwerking het beschermende effect van rauwe melk tenietdoet. Melkverwerking heeft een aanzienlijk effect op de samenstelling van de melk,

waardoor bewerkte, commerciële, koemelk in veel opzichten verschilt van rauwe koemelk. Commerciële melk wordt tegenwoordig vaak gestandaardiseerd, om het gewenste vetgehalte te bereiken, gehomogeniseerd om de scheiding van een vetlaag te voorkomen en de houdbaarheid van de melk te verlengen en verhit om mogelijk aanwezige ziekteverwekkers te vernietigen. Alhoewel deze verwerkingsstappen ervoor zorgen dat melk ten alle tijden beschikbaar is, hebben ze mogelijk ook nadelige effecten. Het verhitten van melk vernietigt namelijk niet alleen ziekteverwekkers, maar ook gunstige bacteriën die een positief effect op de gezondheid kunnen hebben, de zogenoemde probiotica. Daarnaast kan verhitting de structuur van warmtegevoelige melkcomponenten, zoals eiwitten, veranderen waardoor deze hun werking verliezen. Homogenisatie, melk onder hoge druk door nauwe gaatjes persen zodat er kleinere vetbolletjes ontstaan, zorgt verder voor een toename in het zogenoemde melkvetoppervlak. Om dit oppervlak op te vullen zullen melkeiwitten, met name caseïnes en β-lactoglobuline, worden opgenomen. Aangezien dit de belangrijkste allergenen (eiwitten die een allergische reactie kunnen opwekken) in melk zijn, wordt er gedacht dat homogenisatie de allergeenpresentatie aan het afweersysteem verandert. Al deze veranderingen zouden kunnen verklaren waarom het allergie-beschermende effect van rauwe melk verloren lijkt te gaan na melkverwerking.

Ondanks de veelbelovende resultaten van de epidemiologische onderzoeken, bevestigen de waargenomen associaties geen oorzakelijk verband. Zonder deze bevestiging zullen de mogelijke gezondheidsvoordelen nooit opwegen tegen de potentiële risico's en zal de discussie rondom het drinken van rauwe melk blijven voortduren. Om dit eeuwigdurende debat te stoppen zal het epidemiologische bewijs moeten worden ondersteund door een oorzakelijk verband en zullen wetenschappers en de zuivelindustrie moeten samenwerken om in de toekomst microbiologisch veilige alternatieven te kunnen ontwikkelen. De wereldwijde consumptie van koemelk maakt het immers tot een aantrekkelijke preventieve strategie voor allergieën als het risico op infecties kan worden overwonnen.

In dit proefschrift is onderzocht of het huidige epidemiologische bewijs, dat een allergie-beschermend effect van het drinken van rauwe koemelk aantoont, kan worden onderbouwd door een oorzakelijk verband door gebruik te maken van preklinische muismodellen. Daarnaast zijn er verschillende onderzoeksmethoden gebruikt om een beter inzicht te krijgen in de rauwe melkcomponenten die verantwoordelijk zijn voor de beschermende effecten en het onderliggende werkingsmechanisme. Deze kennis zal de ontwikkeling van een veilig én allergie-beschermend product in de toekomst kunnen bevorderen en zal daarnaast antwoord geven op de ondertitel van dit proefschrift 'well done, medium or rare'?

De meeste epidemiologische onderzoeken hebben een beschermend effect van rauwe melk op astma aangetoond. In **Hoofdstuk 3** is er daarom gebruik gemaakt van een huisstofmijt-geïnduceerd allergisch astma muismodel om te onderzoeken of de waargenomen associaties konden worden onderbouwd door een oorzakelijk verband. Huisstofmijt is een allergeen dat bij tenminste 85% van de astmapatiënten klachten veroorzaakt. Door dit allergeen in de luchtwegen toe te dienen, werden de muizen hier gevoelig voor gemaakt. In overeenstemming met het bestaande epidemiologische bewijs voorkwam rauwe koemelk de ontwikkeling van allergisch astma. Beschermende effecten werden waargenomen op zowel de luchtwegreactiviteit als op luchtwegontsteking; rauwe melk voorkwam een verhoging in luchtwegreactiviteit en verlaagde het aantal ontstekingscellen (eosinofielen, neutrofielen, lymfocyten en macrofagen) in de long. Deze effecten vielen samen met een verlaging van de T helper 2 cel (Th2) reactie (geassocieerd met allergie).

Naast astma, laten de resultaten van **Hoofdstuk 4** zien dat rauwe koemelk ook bescherming biedt tegen het ontstaan van voedselallergie. Muizen die gedurende acht dagen oraal werden blootgesteld aan rauwe melk, voordat ze gevoelig werden gemaakt voor het kippenei-eiwit ovalbumine (OVA), vertoonden minder allergische symptomen na provocatie met OVA. De allergische huidreactie na OVA injectie in het oor en de anafylactische shocksymptomen werden verlaagd en de door anafylaxie veroorzaakte daling in lichaamstemperatuur bleef uit. Dit voedselallergie-beschermende effect werd aangetoond in vier verschillende onderzoeken (**Hoofdstukken 4-7**), waarin gebruik werd gemaakt van vier verschillende rauwe melk batches. Deze resultaten benadrukken de consistente werkzaamheid van rauwe koemelk.

Het feit dat rauwe melk zowel de ontwikkeling van allergisch astma als van voedselallergie kon voorkomen geeft aan dat rauwe melk het vermogen heeft om immunologische tolerantie te induceren tegen een niet-gerelateerd, niet in melk aanwezig, allergeen. Dit betekent dat rauwe melk het immuunsysteem op zo'n manier kan beïnvloeden dat er geen ongewenste reactie plaatsvindt tegen onschuldige allergenen. Deze immunomodulerende capaciteit van rauwe melk doet vermoeden dat rauwe melk bescherming kan bieden tegen een breed scala aan allergenen; van aëro-allergenen (allergenen aanwezig in de lucht), zoals huisstofmijt en diverse pollen, tot voedselallergenen zoals OVA en pinda. Bovendien wijzen de verkregen resultaten op een nog bredere toepassing, bijvoorbeeld voor andere immuun-gerelateerde aandoeningen zoals auto-immuunziekten en diabetes type 2.

Naast het aantonen van een allergie-beschermend effect van rauwe melk, laten de bestaande epidemiologische onderzoeken ook een verlies aan bescherming zien na melkverwerking. In tegenstelling tot rauwe melk konden gekookte rauwe melk en bewerkte, commerciële, melk het risico op allergieën niet verminderen. In **Hoofdstuk 3** en **Hoofdstuk 4** werden ook deze epidemiologische bevindingen bevestigd. Verhitte rauwe melk (10 min, 80 °C)

vertoonde geen astma-beschermend effect en commerciële melk (zowel gehomogeniseerd als gepasteuriseerd) bood geen bescherming tegen symptomen van voedselallergie. Deze resultaten geven aan dat hoewel melkverwerking geen effect heeft op de voedingswaarde van de melk, het wel schadelijk is voor zijn allergie-beschermende capaciteit.

Het vergelijken van de allergie-beschermende capaciteit van rauwe koemelk met een commerciële melk (door de meeste mensen gedronken), zoals gedaan in **Hoofdstuk 4**, was een logische eerste stap om het huidige epidemiologische bewijs te bevestigen. Deze melksoorten verschilden echter in meerdere aspecten, onder andere in herkomst, vetgehalte en warmtebehandeling, waardoor het moeilijk is om meer inzicht te krijgen in de betrokken rauwe melkcomponenten. In **Hoofstuk 5** werd daarom melk van dezelfde herkomst vergeleken, die slechts verschilde in één verwerkingsstap. De resultaten uit dit hoofdstuk laten zien dat de capaciteit van rauwe melk om de allergische reactie tegen OVA te verminderen behouden blijft na het afromen van de melk (het verwijderen van de vetlaag), maar verloren gaat na het pasteuriseren van de melk, wat aangeeft dat niet het vetgehalte, maar de warmtegevoelige melkcomponenten ten grondslag liggen aan het allergie-beschermende effect. Bovendien benadrukken deze resultaten dat ook relatief lage temperaturen, zoals gebruikt tijdens pasteurisatie (70-80 °C gedurende 15-20 seconden), schadelijk kunnen zijn voor het allergie-beschermende effect.

De warmtegevoelige wei-eiwit fractie wordt vaak gerelateerd aan de allergie-beschermende effecten van rauwe koemelk. Naast α-lactalbumine, β-lactoglobuline en runderalbumine (de belangrijkste allergenen in de wei-eiwit fractie), is deze fractie namelijk rijk aan eiwitten met immunomodulerende eigenschappen zoals immunoglobulinen, lactoferrine, enzymen en cytokines. Warmtebehandeling van de melk kan ervoor zorgen dat deze immunomodulerende eiwitten hun biologische activiteit verliezen (zoals is samengevat in **Hoofdstuk 2**). Om een beter inzicht te krijgen in de relatie tussen het verlies van wei-eiwitten door warmtebehandeling en allergieontwikkeling, is in **Hoofdstuk 6** het wei-eiwit profiel van rauwe koemelk vergeleken met dat van melk verhit op verschillende temperaturen (50-80 °C). Veranderingen in het wei-eiwit profiel werden vervolgens gerelateerd aan de capaciteit van de melk om OVA-geïnduceerde voedselallergie te voorkomen. Zoals verwacht nam het aantal wei-eiwitten af naarmate de verhittingstemperatuur toe nam. Een aanzienlijk verlies in wei-eiwitten werd waargenomen vanaf 75 °C; met name α-lactalbumine, β-lactoglobuline en runderalbumine denatureerden vanaf deze temperatuur. Uit een vervolganalyse die zich uitsluitend richtte op wei-eiwitten met immunomodulerende eigenschappen bleek echter dat deze eiwitten al denatureerden vanaf 65 °C. Dit is in overeenstemming met eerdere onderzoeken die hebben laten zien dat met name de bioactieve eiwitten in de wei-eiwit fractie warmtegevoelig zijn.

Interessant is dat het verlies van immunologisch actieve wei-eiwitten samenviel met het verlies van de allergie-beschermende capaciteit van melk in het OVA-geïnduceerde voedselallergiemodel. Het allergie-beschermende effect dat werd waargenomen wanneer muizen werden behandeld met rauwe melk hield stand tot een temperatuur van 60 °C. Melk die gedurende 30 minuten op een temperatuur boven de 60 °C werd verhit (65-80 °C) was niet langer beschermend. Naast het aantonen van het belang van immunologisch actieve wei-eiwitten die denatureren rond de 65 °C, sluiten deze resultaten ook de bijdrage van α-lactalbumine, β-lactoglobuline en runderalbumine uit, aangezien deze eiwitten denatureren bij temperaturen boven de 75 °C. Een nadere blik op de individuele wei-eiwitten toonde een significante afname in de concentratie van complement C7, monocyt differentiatie antigeen CD14 en polymere immunoglobulinereceptor bij 65 °C. De significante afname in deze eiwitten kan (gedeeltelijk) het verlies aan bescherming bij dezelfde temperatuur verklaren. Andere immunologisch actieve wei-eiwitten (bijvoorbeeld immunoglobulinen en lactoferrine) lieten echter ook een geleidelijke afname rond de 65 °C zien. Vanwege de vele immunologisch actieve wei-eiwitten aanwezig in rauwe melk, kunnen zelfs kleine veranderingen in elk van hen de uiteindelijke allergische reactie beïnvloeden. Uiteindelijk ligt het gezamenlijke effect van veranderingen in verschillende wei-eiwitten tegelijkertijd waarschijnlijk ten grondslag aan het verlies van allergiebescherming.

Een andere manier om meer inzicht te krijgen in de rauwe melkcomponenten die verantwoordelijk zijn voor het allergie-beschermende effect is het fractioneren van de melk op basis van grootte. In **Hoofdstuk 9** zijn deze verschillende rauwe melkfracties getest op hun vermogen om de allergische reactie op celniveau te beïnvloeden. De resultaten toonden aan dat de componenten die bijdragen aan het allergie-beschermende effect van rauwe melk waarschijnlijk een molecuulgewicht hebben van > 37 kDa. Aangezien α-lactalbumine en β-lactoglobuline een molecuulgewicht hebben van < 25 kDa, is dit nog een indicatie dat deze wei-eiwitten niet verantwoordelijk zijn voor de beschermende effecten. Karakterisatie van de beschermende fracties zal uiteindelijk meer inzicht moeten geven in de daadwerkelijke rauwe melkeiwitten die betrokken zijn bij het allergie-beschermende effect.

Naast de betrokken rauwe melkcomponenten, zal ook een betere kennis van het werkingsmechanisme dat ten grondslag ligt aan de allergie-beschermende effecten van rauwe melk de ontwikkeling van microbiologisch veilige alternatieven bevorderen. De huidige literatuur speculeert met name over de vorming van een beschermende omgeving waardoor het immuunsysteem niet reageert op onschuldige allergenen. De resultaten van **Hoofdstuk 5** laten inderdaad zien dat de beschermende effecten van rauwe melk gepaard gaan met een verhoging in celtypes die geassocieerd worden met immunologische tolerantie zoals regulatoire T cellen en $CD103^+$ dendritische cellen. In **Hoofdstuk 4** werd duidelijk dat epigenetische regulatie (veranderingen in de uitwerking van genen zonder dat het DNA

veranderd) hier mogelijk ten grondslag aan ligt. Blootstelling aan rauwe melk verhoogde namelijk de histonacetylatie (bepaalde markeringen rondom het DNA) van FoxP3; een belangrijke transcriptiefactor in de ontwikkeling van regulatoire T cellen. Naast FoxP3, beïnvloedde rauwe melk ook de histonacetylatie van verschillende andere T cel-gerelateerde genen. Het aangrijpen van histon-markeringen op T cellen zou daarom mogelijk ten grondslag kunnen liggen aan de allergie-beschermende effecten van rauwe koemelk.

Behalve het moduleren van de T cel reactie, laat **Hoofdstuk 9** zien dat rauwe melk ook in staat is om de activering van mestcellen te remmen. **Hoofdstuk 3** liet zien dat de onderdrukking van lokale, pulmonale, type 2 cytokine concentraties (IL-5 en IL-13; belangrijke ontstekingsstimulerende stoffen in de ontwikkeling van allergisch astma), geproduceerd door andere immuuncellen dan T cellen, cruciaal leek te zijn voor de door rauwe melk-geïnduceerde bescherming tegen allergisch astma. Aangezien het bekend is dat ook mestcellen en basofielen deze type 2 cytokines kunnen produceren duidde dit op een mogelijk effect van deze effectorcellen in het allergie-beschermende effect. Het blootstellen van mestcellen aan rauwe melk verminderde inderdaad hun activatie zoals aangetoond door een verminderde β-hexosaminidase afgifte en een verlaagde IL-6 en IL-13 productie.

Dit proefschrift richtte zich vooral op het allergie-beschermende karakter van rauwe melk en toonde zijn capaciteit om zowel allergisch astma als voedselallergie te voorkomen. Dit beschermende effect werd duidelijk vernietigd door melkverwerking; hoogstwaarschijnlijk vanwege een verlies van functionaliteit van immunomodulerende eiwitten aanwezig in de wei-eiwit fractie van de melk. In **Hoofstuk 8** is onderzocht of melkverwerking ook een negatief effect heeft op de allergeniciteit van de melk, dat wil zeggen, het vermogen van de melk om een allergische reactie te veroorzaken. Zowel rauwe melk, als de wei-eiwit fractie geïsoleerd uit rauwe melk bleken inderdaad een lagere allergeniciteit te hebben dan hun verwerkte tegenhangers. Deze bevindingen werden uitgebreid getoond in een muismodel voor koemelkallergie en werden daarnaast bevestigd in een provocatietest waarin koemelk-allergische kinderen rauwe koemelk tot het maximale niveau van 50 mL konden verdragen, terwijl de provocatie met bewerkte, commerciële melk in de meeste gevallen eerder moest worden gestopt vanwege de ontwikkeling van allergische symptomen.

Wanneer rauwe melk wordt geproduceerd onder strenge hygiëne voorschriften en microbiologische criteria, brengt het drinken ervan weinig risico's met zich mee. Desalniettemin kan een nul risico nooit worden gegarandeerd en daarom wordt de consumptie van rauwe koemelk sterk afgeraden of zelfs verboden door overheidsinstanties. De resultaten van zowel **Hoofdstuk 5** als **Hoofdstuk 7** laten zien dat het verrijken van bewerkte melk met bioactieve wei-eiwitten een veelbelovend alternatief zou kunnen zijn om allergieën te bestrijden. Zowel alkalische fosfatase, lactoferrine, immunoglobuline G en osteopontine lieten beschermende

effecten zien in het OVA-geïnduceerde voedselallergiemodel, maar het bleek de combinatie van deze vier bioactieve wei-eiwitten te zijn die het meest effectief was waardoor het belang van de melkmatrix werd benadrukt. Door mildere verwerkingsmethoden te gebruiken, die ziekteverwekkers verwijderen maar de gunstige rauwe melkcomponenten behouden, zou deze rauwe melkmatrix behouden kunnen blijven. Het filtreren van rauwe melk is zo'n methode en werd toepast om de wei-eiwit fractie te isoleren die gebruikt is in **Hoofdstuk 8** van dit proefschrift. Deze wei-eiwit fractie bleek een lagere allergeniciteit te hebben in vergelijking met verwerkte wei-eiwitten. Aangezien wei-eiwitten het hoofdbestandsdeel zijn van veel zuigelingenvoeding in Nederland, kan het gebruik van 'natieve' wei-eiwitten enorme mogelijkheden bieden.

Samenvattend levert dit proefschrift wetenschappelijk onderbouwd bewijs dat het allergie-beschermende effect van rauwe koemelk bevestigt, zoals eerder aangetoond door epidemiologische onderzoeken. Voor het eerst kon een oorzakelijk verband worden aangetoond door gebruik te maken van preklinische muismodellen. Rauwe koemelk voorkwam zowel astma als voedselallergie en dit beschermende effect werd duidelijk vernietigd door melkverwerking. De bevindingen van dit proefschrift laten verder zien dat warmtegevoelige wei-eiwitten die denatureren rond de 65 °C hoogstwaarschijnlijk verantwoordelijk zijn voor het allergie-beschermende effect van rauwe melk en dat zowel het moduleren van de T cel reactie als het remmen van de allergische effector reactie ten grondslag liggen aan de beschermende effecten. Naast een allergie-beschermend effect, bleken rauwe melk en 'natieve' wei-eiwitten ook een lagere allergeniciteit te hebben in vergelijking met hun verwerkte tegenhangers.

Concluderend mag het duidelijk zijn dat de huidige industriële melkverwerkingsmethoden (dat wil zeggen, standaardisatie, homogenisatie en warmtebehandeling) schadelijk zijn voor de allergie-beschermende effecten van rauwe koemelk; niets veranderen en blijven bij 'well done' is daarom geen mogelijkheid. De rauwe melk alleen verwarmen, als middenweg ('medium'), is ook geen optie als er temperaturen boven de 60 °C worden gebruikt. Daarentegen kan het verrijken van bewerkte melk met bioactieve wei-eiwitten ook worden gezien als 'medium' en is er aangetoond dat dit een veelbelovend alternatief zou kunnen zijn voor rauwe melk. Het feit dat de momenteel beschreven associaties tussen het drinken van rauwe melk en de bescherming tegen verschillende allergieën werden bevestigd door een oorzakelijk verband pleit echter duidelijk voor 'rare', en de sterkte en overtuiging van de bevindingen benadrukken dat er gestreefd moet worden naar een schonere melkproductie. Aangezien het potentiële risico op infecties altijd een tegenargument zal blijven, moet het verwarmen van rauwe melk tot een maximale temperatuur van 60 °C ook als 'rare' worden beschouwd, zolang de warmtegevoelige wei-eiwitten maar behouden blijven.

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ABOUT THE AUTHOR

Suzanne Abbring was born on October 12, 1989 in Hoorn, the Netherlands. After graduating from high school (RSG, Enkhuizen) in 2008, she moved to Wageningen to study Nutrition and Health at the Wageningen University. She received her Bachelor's degree with a minor in Communication in 2011 and continued with the Master Nutrition and Health (specialization Molecular Nutrition and Toxicology). As part of the Master program, Suzanne conducted her thesis at the Nutrition and Pharmacology group under the supervision of prof. dr. Renger Witkamp and dr. Jocelijn Meijerink. Here, she studied the effects of docosahexaenoyl-serotonin on the production of CCL20 and other cytokines in human peripheral blood mononuclear cells. Thereafter, Suzanne moved to Southampton, United Kingdom, to conduct a six-month internship at the Nutrition and Metabolism research group led by prof. dr. Philip Calder at the University of Southampton. During this internship, she assessed the effects of omega-3 fatty acids on postprandial inflammation in normal weight and obese subjects. During her studies, Suzanne was actively involved in study association Di-et-Tri and she joined the board as treasurer in 2010-2011.

After successfully obtaining her Master's degree in 2013, Suzanne joined the division of Pharmacology within the Utrecht Institute for Pharmaceutical Sciences in 2014 as junior researcher. Under the supervision of Aletta Kraneveld, she conducted various research projects in the immunopharmacology field. In 2015, Suzanne started as a PhD candidate in the same research group under the supervision of prof. dr. Johan Garssen and dr. Betty van Esch, investigating the potential of raw, unprocessed, cow's milk to target allergic diseases. The results of this work are described in this thesis.

As part of her PhD, Suzanne was trained in the PhD program Drug Innovation of the Graduate School of Life Sciences and completed several courses in Immunology, Statistics, and Transferable Skills. She received a travel grant from the European Academy of Allergy and Clinical Immunology (EAACI) to attend the annual congress in Helsinki, Finland in 2017 and was awarded with the best poster prize at the EAACI annual congress in Vienna, Austria (in two different sessions, 2016) and Helsinki, Finland (2017) and at the EAACI Immunology Winter School in Trysil, Norway (2019). Suzanne furthermore represented Utrecht University at the FIGON PhD Student Competition in Ede (3rd place, 2018). As of July 2019, Suzanne started as a postdoctoral researcher at the division of Pharmacology (Utrecht University) to continue her work on the allergy-protective effects of raw cow's milk consumption.

LIST OF PUBLICATIONS

Abbring S[*], Xiong L[*], Diks MAP, Baars T, Garssen J, Hettinga K[†], van Esch BCAM[†]. Loss of allergy-protective capacity of raw cow's milk after heat treatment coincides with loss of immunologically active whey proteins – Native proteomics combined with a functional readout for allergic diseases. *Submitted for publication*.

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Abbring S, Blokhuis BRJ, Miltenburg JL, Romano Olmedo KGJ, Garssen J, Redegeld FAM, van Esch BCAM. Direct inhibition of the allergic effector response by raw cow's milk – An extensive *in vitro* assessment. *Submitted for publication*.

Abbring S, Wolf J, Ayechu Muruzabal V, Diks MAP, Alashkar Alhamwe B, Alhamdan F, Harb H, Renz H, Garn H, Garssen J, Potaczek DP, van Esch BCAM. Raw cow's milk reduces allergic symptoms in a murine model for food allergy – A potential role for epigenetic modifications. *Nutrients* (2019) 11(8): 1721. doi: 10.3390/nu11081721.

Abbring S, Ryan JT, Diks MAP, Hols G, Garssen J, van Esch BCAM. Suppression of food allergic symptoms by raw cow's milk in mice is retained after skimming but abolished after heating the milk – A promising contribution of alkaline phosphatase. *Nutrients* (2019) 11(7): 1499. doi: 10.3390/nu11071499.

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(7): 1013-25. doi: 10.1111/cea.13399.

Abbring S, Hols G, Garssen J, van Esch BCAM. Raw cow's milk consumption and allergic diseases – The potential role of bioactive whey proteins. *Eur J Pharmacol* (2019) 843: 55-65. doi: 10.1016/j.ejphar.2018.11.013.

Abbring S[*], Verheijden KAT[*], Diks MAP, Leusink-Muis A, Hols G, Baars T, Garssen J, van Esch BCAM. Raw cow's milk prevents the development of airway inflammation in a murine house dust mite-induced asthma model. *Front Immunol* (2017) 8: 1045. doi: 10.3389/fimmu.2017.01045.

[*] *These authors have contributed equally to this work*

Wu J, de Theije CGM, da Silva SL, **Abbring S**, van der Horst H, Broersen LM, Willemsen L, Kas M, Garssen J, Kraneveld AD. Dietary interventions that reduce mTOR activity rescue autistic-like behavioral deficits in mice. *Brain Behav Immun* (2017) 59: 273-87. doi: 10.1016/j.bbi.2016.09.016.

van Esch BCAM, **Abbring S**, Diks MAP, Dingjan GM, Harthoorn LF, Vos AP, Garssen J. Post-sensitization administration of non-digestible oligosaccharides and *Bifidobacterium breve* M-16V reduces allergic symptoms in mice. *Immun Inflamm Dis* (2016) 4(2): 155-65. doi: 10.1002/iid3.101.