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Review

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.

1. 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 (European Academy of Allergy and Clinical Immunology, 2014). In the EU, the estimated health care costs to manage allergic diseases range between 55 and 151 billion euro (Zuberbier et al., 2014). 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 (Strachan, 1989; Holgate and Polosa, 2008). In line with this 'hygiene hypothesis' several epidemiological studies have shown a protective 'farm effect' on allergic diseases (von Mutius and Vercelli, 2010). 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 (Braun-Fahrlander et al., 1999; Kilpelainen et al., 2000; Riedler et al., 2000; von Ehrenstein et al., 2000; Alfven et al., 2006). 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 (Riedler et al., 2001; Remes et al., 2003; Perkin and Strachan, 2006; Ege et al., 2007; Waser et al., 2007). The latter is of particular interest, since its protective effect was found to be independent of farm-related co-exposures (Riedler et al., 2001; Perkin and Strachan, 2006; Waser et al., 2007). 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 (Abbring et al., 2017).

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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* (Oliver et al., 2009; Claeys et al., 2013). The risks of disease outbreaks that could be caused by these pathogens are the basis for governmental agencies to prohibit the consumption of raw cow's milk, especially for pregnant women, infants and children (Committee on Infectious Diseases and Committee on Nutrition and American Academy of Pediatrics, 2014). 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 (Baars, 2013).

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 (Loss et al., 2011; Brick et al., 2016; Abbring et al., 2017). 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.

2. 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) (Chandan, 2011). 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) (Claeys et al., 2013; Verhoeckx et al., 2015). 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 (Rankin et al., 2010). 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 (Michalski and Januel, 2006). 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) (Perkin, 2007). 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 (Poulsen et al., 1987, 1990), but these findings could not be confirmed in clinical studies (Host and Samuelsson, 1988; Pelto et al., 2000). 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 (2007).

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 (Guetouache et al., 2014). 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 (Braun-Fahrlander and von Mutius, 2011). 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.

3. 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' (Schrezenmeir et al., 2000). 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 (Perdijk et al., 2018). 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).

3.1. 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 (Mehra et al., 2006). The predominant immunoglobulin in bovine milk is IgG, but IgA and IgM are also present (van Neerven et al., 2012).

A direct link between IgG and allergies was observed in a murine model were 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 (Mosconi et al., 2010). 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 (Collins et al., 1991; van Neerven et al., 2012). These allergens might also be present in small amounts in bovine

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	(Collins et al., 1991; Ober et al., 2001; Mosconi et al., 2010; van Neerven et al., 2012)
	• IgG-mediated blocking	(Malbec and Daëron, 2007; Renz et al., 2018)
	• Receptor-mediated inhibition	(Daëron et al., 1995; Burton et al., 2014; Burton et al., 2018)
	• Bacteria and virus clearance	(Yolken et al., 1985; Li-Chan et al., 1994; Korhonen et al., 2007)
Lactoferrin	• Antimicrobial activity	(Giansanti et al., 2016; Munblit et al., 2017)
	• Outgrowth of *Bifidobacteria* and *Lactobacilli*	(Tian et al., 2010; Oda et al., 2014; Giansanti et al., 2016)
	• Stimulation of TGF-β and IL-10 production in the gut	(Takakura et al., 2006; Lonnerdal et al., 2011; Liao et al., 2012)
TGF-β	• Improvement of intestinal barrier function	(Planchon et al., 1994; Roche et al., 2000; Howe et al., 2005; Kotler et al., 2013)
	• Induction of different subclasses of regulatory T cells(FoxP3^{+} regulatory T cells, Tr1 cells, Th3 cells)	(Chen et al., 2003; Faria and Weiner, 2005; Kretschmer et al., 2005; Maynard et al., 2007)
	• IgA class switching	(Faria and Weiner, 2005)
IL-10	• Inhibition of APC function	(de Waal Malefyt et al., 1991; Buelens et al., 1997)
	• Inhibition of mast cell activation, Th2 cell activation and eosinophil function	(Takanaski et al., 1994; Grunig et al., 1997; Royer et al., 2001)
	• IgG class switching	(Jeannin et al., 1998)
	• Induction of Tr1 cells	(Levings et al., 2005)
Alkaline phosphatase	• Detoxification of bacterial LPS	(Lalles, 2010)
Osteopontin	• Modulation of Th1/Th2 immune responses?	(Nau et al., 1999; Konno et al., 2005; Renkl et al., 2005; Xanthou et al., 2007; Nagasaka et al., 2008; Kurokawa et al., 2009)
	• Carrier protein lactoferrin	(Yamniuk et al., 2009)

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

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 (van Neerven et al., 2012). 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 (Ober et al., 2001).

Interestingly, bovine IgG was also shown to bind to a wide range of human pathogenic bacteria and viruses (Yolken et al., 1985; Li-Chan et al., 1994; Korhonen et al., 2007). It binds for example to human respiratory syncytial virus (RSV) and enhances its phagocytosis via FcγRII receptors on macrophages, neutrophils and monocytes (den Hartog et al., 2014). 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 (Pérez-Yarza et al., 2007; Régnier and Huels, 2013). 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 (Malbec and Daëron, 2007). 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 (Renz et al., 2018). In the second mechanism, the allergen simultaneously binds to IgE and IgG antibodies present on the surface of mast cells which induces crosslinking 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 (Daëron et al., 1995; Burton et al., 2014, 2018). 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) (Renz et al., 2018). 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.

3.2. Lactoferrin

Lactoferrin is produced and released by mucosal epithelial cells into most exocrine fluids, and particularly into milk (Cacho and Lawrence, 2017). 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 (Giansanti et al., 2016; Munblit et al., 2017). 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 (Fischer et al., 2006; Legrand, 2012, 2016).

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* (Tian et al., 2010; Oda et al., 2014; Giansanti et al., 2016). 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 (Björkstén et al., 2001; Kalliomäki et al., 2001; Sjögren et al., 2009). 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 (Tan et al., 2014; McKenzie et al., 2017; Wang et al., 2018). A direct link between lactoferrin and allergic diseases is, to our knowledge, only investigated by one study. Kruzel et al. (2006) showed that lactoferrin reduces pollen antigen-induced airway inflammation in a murine asthma model. 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 (Shin et al., 2008; Liao et al., 2012). 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 (Lonnerdal et al., 2011). Moreover, human monocyte-derived dendritic cells (DCs) differentiated in the presence of bovine lactoferrin showed a tolerogenic phenotype (Puddu et al., 2011).

Bovine lactoferrin has already been shown to be protective against respiratory tract infections in infants when added to infant formula (Chen et al., 2016). 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.

3.3. 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 (Chatterton et al., 2013). 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 (Rautava and Walker, 2009).

TGF-β is a multifunctional cytokine which plays a key role in the development and maturation of the mucosal immune system (Oddy and Rosales, 2010). 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* (Planchon et al., 1994; Roche et al., 2000; Howe et al., 2005). These protective effects were, at least partially, induced by increased expression of intestinal tight junctions leading to improved barrier function (Kotler et al., 2013). 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 (Groschwitz and Hogan, 2009). The effect of TGF-β1 on intestinal barrier function could explain the observed protection of TGF-β against allergies in infancy and early childhood (Oddy and Rosales, 2010).

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 (Scott et al., 2011). The crucial role of TGF-β in the induction of immune tolerance is illustrated by, for example, Verhasselt et al. (2008) 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. In agreement with these studies, Penttila et al. (2003) 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. 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. Down-regulation of the allergic response was maintained when TGF-β was no longer present in the diet (Penttila, 2006).

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 (Chen et al., 2003). This conversion could not take place when TGF-β signaling was deficient, showing the necessity of TGF-β induced FoxP3 expression (Kretschmer et al., 2005). 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) (Faria and Weiner, 2005; Maynard et al., 2007). 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 (Perez-Machado et al., 2003).

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 (Faria and Weiner, 2005). In epidemiological studies, IgA deficiency was associated with infections and allergic diseases during childhood (Janzi et al., 2009). In addition, low levels of human milk IgA correlated with allergy development (Jarvinen et al., 2000; Savilahti et al., 2005). 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 (Lonnerdal et al., 2011; Liao et al., 2012). 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.

3.4. IL-10

Just like TGF-β, IL-10 is a regulatory cytokine present in bovine milk although in much lower concentrations (van Neerven et al., 2012). 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 (Hawrylowicz and O'Garra, 2005). It, for example, inhibits IgE-induced mast cell activation, Th2 cell activation and eosinophil function (Takanaski et al., 1994; Grunig et al., 1997; Royer et al., 2001). It also inhibits antigen-presenting cell (APC) function by reducing the expression of MHCII and co-stimulatory molecules and by preventing DC maturation (de Waal Malefyt et al., 1991; Buelens et al., 1997). IL-10 furthermore enhances immunoglobulin class switching in B cells and it possibly induces IL-10 secreting regulatory T cells (Jeannin et al., 1998; Levings et al., 2005). In line with these findings there is evidence for an inverse correlation between IL-10 and allergic diseases (Borish et al., 1996; Heaton et al., 2005).

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 (den Hartog et al., 2011). 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 (den Hartog et al., 2011). Bovine IL-10 was furthermore shown to be equally effective in inhibiting human DC activation as human IL-10 (den Hartog et al., 2011). 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 (Faria and Weiner, 2005; Pletinckx et al., 2011).

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) (Takakura et al., 2006). The tolerogenic feature of these cytokines could contribute to the observed allergy protection by raw cow's milk consumption.

3.5. 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 (Rankin et al., 2010). 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 (Lalles, 2010). The tissue non-specific ALP isoform is present in raw milk (Lalles, 2016). About 30% of this ALP is bound to the fat fraction, while the remaining part is in the milk serum (Morton, 1953).

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 (Lalles, 2010). 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 (Beumer et al., 2003; Tuin et al., 2009; Whitehouse et al., 2010). 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 (Bender et al., 2015).

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 (Iyngkaran et al., 1995; Tuin et al., 2009; Whitehouse et al., 2010). 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.

3.6. 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 (Demmelmair et al., 2017). OPN is encoded by one gene but several isoforms exist as a result of post-translational modifications. These modifications vary greatly between cell types and are responsible for the diverse biological functions of OPN (Frenzel and Weiss, 2011).

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 (Renkl et al., 2005). In addition, OPN-deficient mice show insufficient Th1 immunity when infected with *Mycobacterium bovis* BCG (Nau et al., 1999). Levels of OPN were furthermore found to be elevated in several Th1-associated diseases, such as rheumatoid arthritis, multiple sclerosis, Crohn's diseases and tuberculosis (Koguchi et al., 2003; Comabella et al., 2005; Sato et al., 2005; Xu et al., 2005).

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 (Konno et al., 2005). 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 (Kurokawa et al., 2009). 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 (Xanthou et al., 2007; Nagasaka et al., 2008). 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 (Xanthou et al., 2007). A relation between OPN and allergic diseases was furthermore demonstrated by recent findings showing increased OPN expression in Th2-related diseases, like asthma (Samitas et al., 2011).

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 (Jiang and Lonnerdal, 2016). 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 (Yamniuk et al., 2009). This illustrates that besides a possible direct allergy-protective effect, OPN can also indirectly influence allergic outcomes.

4. 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 (Desmasures et al., 1997; Perkin, 2007). Raw milk was found to contain more bacteria, but also higher levels of bacterial endotoxins compared to pasteurized milk (Suhren et al., 1986; Sipka et al., 2015). 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 (Braun-Fahrlander et al., 2002). 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 (Sipka et al., 2015). Gehring et al. (2008) 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. 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 cells 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 (Loss et al., 2011). 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. (2014).

5. Passage through the 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 gastrointestinal (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) (Oddy and Rosales, 2010). 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 (Annes et al., 2003). 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 (Penttila et al., 2003; Ando et al., 2007). Comparable results have been observed for IL-10 (Slavin et al., 2001).

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 (Troost et al., 2001). 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 (Davidson and Lonnerdal, 1987). 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) (Dallas et al., 2012). 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 (Britton and Koldovsky, 1987). 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 (Yamniuk et al., 2009). 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 (Giansanti et al., 2016).

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 (Jasion and Burnett, 2015). 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 (Ulfman et al., 2018). 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 (Jasion and Burnett, 2015). 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 (Payne, 1969).

Oral administration of bovine ALP is shown to be protective in experimental models for inflammatory diseases (Lalles, 2014). Many of these models focus on intestinal inflammation, suggesting that bovine ALP can withstand the harsh conditions in the GI tract (Tuin et al., 2009; Bol-Schoenmakers et al., 2010; Whitehouse et al., 2010). 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 (Martinez-Moya et al., 2012). 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 (Jiang and Lonnerdal, 2016). Just as demonstrated for lactoferrin, this indicates that partial digestion of OPN is not necessarily detrimental for its biological functions.

6. 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 (Loss et al., 2011; Brick et al., 2016; Abbring et al., 2017). Of all raw milk components, mainly whey proteins are susceptible to heat treatment (Brick et al., 2017). 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 (Brick et al., 2017). At which temperature this happens depends on the protein, but generally it is assumed that whey proteins denature above 65 °C (Verhoeckx et al., 2015). 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 (Mainer et al., 1997; Korhonen et al., 2000). 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 (Li-Chan et al., 1995). This is more comparable to results observed in human milk, where IgG levels decreased with about 60% after pasteurization (Adhisivam et al., 2018). In addition, when measured in commercially available pasteurized milk total IgG levels were much lower compared to raw milk (Li-Chan et al., 1995; Loss et al., 2011).

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 (Schwarcz et al., 2008). 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. (2016) showed only a small reduction in lactoferrin levels upon pasteurization. This small, but significantly reduction was confirmed in commercially available pasteurized shop milk, which contained lower lactoferrin levels than raw milk (Lorenzen et al., 2011). Higher heat treatments (above 80 °C), such as UHT, substantially lowered lactoferrin levels (Brick et al., 2017).

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 (Peroni et al., 2009). TGF-β2 concentrations did not differ between pasteurized and raw milk, but levels were reduced when raw milk was heated to 87 °C (Elfstrand et al., 2002; Loss et al., 2011; Hodgkinson et al., 2014). Similar results were obtained for the TGF-β2 content of the whey protein fraction of milk; levels decreased as heat treatment increased in intensity (Akbache et al., 2011). 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 (Untalan et al., 2009; Ewaschuk et al., 2011). 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 (Rankin et al., 2010). In contrast to ALP, OPN is relatively heat-stable. Pasteurization of bovine milk did not affect OPN concentrations (Zhang et al., 2016). 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 (Yamniuk et al., 2009).

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 (Wijayanti et al., 2014). 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 (Akbache et al., 2011). Moreover, β-lactoglobulin also readily forms aggregates with immunoglobulins (Chatterton et al., 2013). These aggregation reactions significantly affect the biological functionality of the whey proteins involved.

7. 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 (Fig. 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.

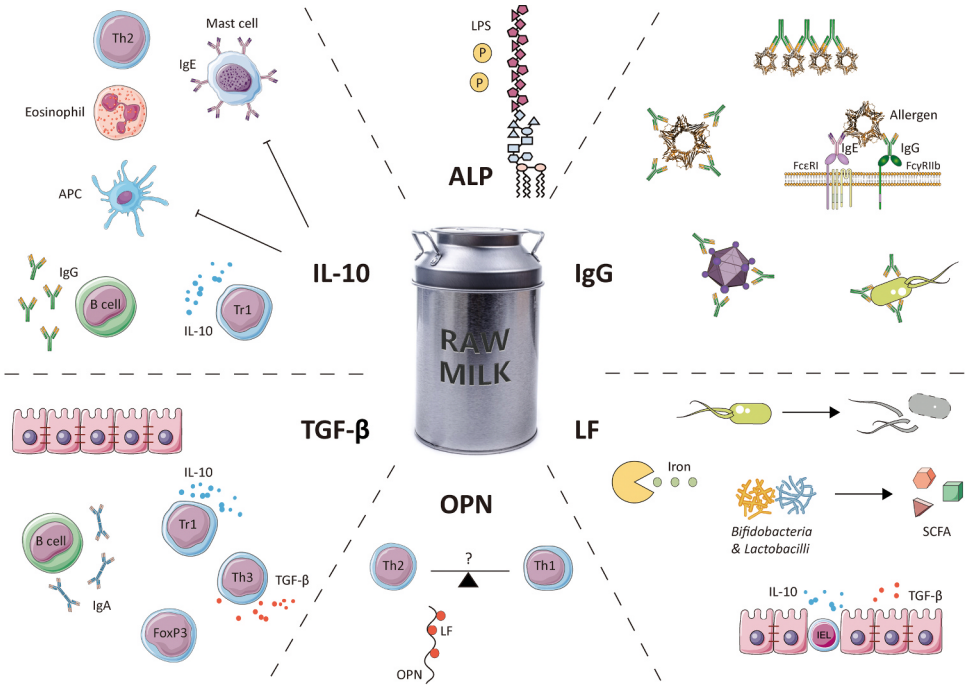

Fig. 1. Graphical representation of the potential allergy-protective effects of raw milk-derived bioactive whey proteins. Shown are whey proteins often mentioned in relation to the allergy-protective effects of raw cow's milk. Bovine IgG antibodies can form immune complexes with allergens. These allergen-IgG 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 phosphatse (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.

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Conflict of interest

This work was financially supported by Danone Nutricia Research. GH, JG, and BvE are (partly) employed at Nutricia Research. All other authors report no potential conflicts of interest.

Author contribution

SA writing - original draft. BvE, GH and JG writing - review & editing.

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