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Review

Milk protein coagulation under gastric conditions: A review

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ABSTRACT

Milk protein coagulation is not only important during the processing of milk into various dairy products, but also during digestion of milk. This review focusses on the gastric coagulation of milk proteins. During this process, coagulation of casein micelles and milk fat globules can occur due to pepsin-induced hydrolysis of the proteins that provide steric stabilisation. The gastric coagulation leads to delayed gastric emptying of casein and fat. Native whey proteins are not susceptible to gastric coagulation or delayed gastric emptying. Both heat treatment and homogenisation of milk lead to weaker gastric curds being formed, which are broken down more easily due to proteolysis and deformation. Incorporation of denatured whey proteins in gastric curds of heated milk delays their emptying. Understanding gastric coagulation and digestion behaviour allows tailoring of gastric transit via compositional differences or processing.

Contents

1. Introduction

When considering the essential role of protein in tissues and processes in the human body, the importance of providing the body with sufficient building blocks, in the form of amino acids, is evident. These are needed to balance daily nitrogen losses, for tissue maintenance and for the synthesis of non-protein nitrogenous substances; furthermore, they also allow additional protein deposition in newly formed tissues for growth (infants and children) and in specific physiological conditions such as pregnancy or lactation. For healthy adults, the protein requirement has been defined as the lowest protein intake that will allow nitrogen equilibrium (i.e., zero nitrogen balance in nitrogen balance studies) and has been experimentally found to be 0.66 g kg^{-1} d^{-1} (= mean protein

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requirement) (WHO, 2007). Based on this, the safe protein requirement has been set to 0.83 g kg^{-1} d^{-1} (= RDA = mean + 2 SD) (WHO, 2007). For other groups of the population, e.g., infants, children, women that are either pregnant or lactation or elderly adults, higher protein requirements have been reported (EFSA, 2012; Ghosh & Uauy, 2016). In addition to protein requirements, specific requirements for each of the 9 indispensable amino acids (IAAs) have been set (EFSA, 2012; Ghosh & Uauy, 2016). Key for sustained maintenance and growth is that both targets for protein and for IAAs are met. Achieving this requires selections of adequate protein sources.

For proteins to be utilised efficiently as sources of amino acids for the body, the protein needs to be digested to free amino acids and di- and tripeptides in the intestine (Moughan & Stevens, 2012). A protein source that allows this is considered a well-digestible protein source, but evidence from literature suggests that notable differences in digestibility of protein sources exists (Bailey & Stein, 2020b; Mathai, Liu, & Stein, 2017). In general, most animal protein sources are characterised by high digestibility (Bailey & Stein, 2020a; Bailey, Mathai, Berg, & Stein, 2020; Hodgkinson, Montoya, Scholten, Rutherfurd, & Moughan, 2018; Mathai et al., 2017) whereas some plant protein sources have much lower digestibility (Bailey & Stein, 2020a; Chalupa-Krebzdak, Long, & Bohrer, 2018; Mathai et al., 2017; Wolfe, Baum, Starck, & Moughan, 2018). Protein quality is often expressed in as the so-called Digestible Indispensable Amino Acid Score (DIAAS), which essentially reflects the ability of the protein to provide sufficient levels of digestible IAAs in proportion to requirements (Wolfe et al., 2018; Wolfe, Rutherfurd, Kim, & Moughan, 2016). Protein sources that score 1.0 or higher on this meet all requirements for digestible IAAs, whereas protein sources that score lower than 1.0 fail to meet requirements for one or more IAAs (Wolfe et al., 2016, 2018). This may be either because of insufficient quantities of the IAAs being present, because of low digestibility, or both.

Among the protein sources that score high on DIAAS and other protein digestibility metrics are the milk proteins (Bos et al., 1999; Calvez et al., 2019; Guillin et al., 2020; Mathai et al., 2017; Schaafsma, 2000). This is hardly surprising, considering that they are the source of protein intended for consumption for all mammalian neonates. Differences in protein composition between different mammals and also throughout lactation are considered to reflect adaptation to the specific requirements of the neonate and development of the digestive system. This is reflected, for instance, in the large differences in casein:whey protein ratio between human and bovine milk, as well as the changes in casein:whey protein ratio throughout lactation. Also unique is that one of the protein fractions in milk, i.e., the casein fraction, is susceptible to coagulation in the stomach, whereas the other fraction, the whey proteins, is not (Boirie et al., 1997). Physiologically, this has been related to the requirement of a controlled transit of protein from the stomach into the intestine to facilitate sustained release of proteins and uptake of amino acids into the bloodstream and maximum utilisation of protein (Soop et al., 2012).

A large proportion of all milk produced world-wide, however, is bovine, buffalo, caprine and ovine milk intended for consumption by humans throughout all stages of life. Also here, coagulation of caseins in the stomach can occur, affecting the gastric transit. Over time, it has become clear that controlling gastric transit of proteins is important in human physiology, and is affected strongly by both the type of milk but also the processing applied (Barbé et al., 2013; Lacroix et al., 2006; Soop et al., 2012). While much work is currently ongoing, the strong foundations for this work were already laid over a century ago.

Combined with developments in understanding of the milk protein structure and interactions, insights have emerged that draw large similarities between physicochemical behaviour of milk proteins under gastric conditions and those encountered using well−known processes such as cheese-making and yoghurt making. In this review, we aim to present these insights and discuss them both in the context of physiological importance, milk protein structure, interactions and digestibility and changes therein as a result of processing. In addition to gastric coagulation, processing of milk can also affect milk protein hydrolysis and biological activity of milk proteins. The former has recently been covered by Dupont and Tomé (2020) while van Lieshout, Lambers, Bragt, and Hettinga (2020) recently covered the latter topic. Thus, these topics will not be our primary focus. In this review, we will focus primarily on bovine milk. Milk from other species and other products prepared from bovine milk are outside the scope of this review.

2. Physiological importance of gastric coagulation of caseins

As outlined earlier, caseins and whey in milk behave very differently under gastric conditions, with the caseins coagulating to form a gastric curd and the whey proteins remaining in solution (Boirie et al., 1997). As a result, whey proteins empty from the stomach into the intestine more rapidly than caseins (Boirie et al., 1997; Mahé et al., 1996). Gastric emptying has been shown to be the major factor controlling the kinetics of nitrogen absorption from milk (Gaudichon et al., 1994). Gastric coagulation is of physiological relevance and ensures a controlled transit of protein through the stomach; this, in turn, ensures a more sustained release of amino acids into the blood following intestinal digestion and absorption (Lacroix et al., 2006; Mahé et al., 1996). In addition, the controlled release of protein to the intestine also ensures that the digestive capacity of the intestine does not become 'overloaded', which is particularly relevant in the case of groups within the population with a reduced digestive or absorption capacity, e.g., infants, elderly adults or people with reduced digestive function due to illness.

Lacroix et al. (2006) showed that in the absence of gastric coagulation, i.e., when using only soluble milk protein, digestion and absorption were too rapid to be sustained in terms of anabolic response and led to increased losses of nitrogen in the form of urea and amino acid through oxidation. Similar effects were also observed by Dangin et al. (2001), who also showed such effects for providing the amino acid composition of casein in the form of free amino acids, rather than intact protein, thus also leading to very rapid gastric transit and absorption. Morens et al. (2003) also showed that increasing milk protein intake increased transfer of dietary nitrogen to urea and did not proportionally increase serum amino acid concentrations, indicating the importance of the splanchic zone in preventing excessive increases in blood amino acid levels due to high protein intake. Net postprandial protein utilisation also decreased with increasing protein intake (Morens et al., 2003). These studies highlight the importance of a controlled release of protein, which is elegantly provided in milk through the combination of whey protein and casein.

When considering gastric coagulation of caseins, it is important to realise that there are notable differences in the extent of gastric coagulation of caseins as well. The physiological importance of firmness of curds formed through gastric coagulation of caseins has become apparent from a large body of work carried out in the first half of the 20th Century, focused around so-called 'soft curd milk' and 'hard curd milk' (see, e.g., Brennemann, 1911; Doan, 1938; Elias, 1932; Hill, 1931). This work, relating to the hardness of the coagulum formed by milk samples under gastric conditions, showed that not only the propensity of caseins to coagulate, but also the type of coagulum formed, was extremely important in the digestive process. Key findings of this work, summarised in Table 1, were

mainly focussed on infant nutrition, but also included examples in adult nutrition. In all cases, consumption of so-called soft curd milk led to reduced digestive difficulties compared with consumption of hard curd milk. Infant feeding studies show that the rate of gastric digestion and stomach clearance is related to curd the hardness of gastric curds formed (Doan, 1938). A gastric emptying study in preterm infants showed gastric emptying rate of 30 mL portions of human milk was considerably fast than of 30 mL portions of infant formula, with an average of with 24.8 mL of human milk having been emptied after one hour (Cavell, 1979). This may be related to the fact that human milk forms softer clots in the stomach than infant formula.

In extreme cases, the appearance of casein curds in stool after consumption of hard curd milk by infants was observed, which were related to the formation of firm coagula in the stomach (Brennemann, 1911; Courtney, 1912; Hess, 1913). The formation of these firm coagula prevents the attachment of proteases on the active sites. As a result, the protected protein clusters could be not broken down into smaller peptides for further intestinal digestion and absorption, leading to a loss of undigested protein in the stools. Analysis showed that protein (~60%), fat (~30%) and ash (~4%) are the main constituents of these curds found in infant stool (Bosworth, 1921; Courtney, 1912). Feeding infants with milk that forms hard curd were associated with undesirable stool consistency and constipation (Doan, 1938; Hill, 1923, 1931). The presence of longer peptides and protein curds in the distal ileum and colon could pose additional microbiological and immunological complications. Protein fermentation by gut microbiota produces unfavourable metabolites, e.g., branched-chain fatty acids, ammonia, phenolic and indolic compounds, biogenic amines, hydrogen sulphide, and nitric oxide (Gilbert, Ijssennagger, Kies, & van Mil, 2018). These proteolytic metabolites have been shown to increase inflammatory response and disrupt gut barrier function (Diether & Willing, 2019). Protein indigestibility is also associated with gastrointestinal symptoms in infants such as bloating, diarrhoea and colic (van de Heijning, Berton, Bouritius, & Goulet, 2014).

Due to limited analytical resources available in the first half of the 20th Century, it was unfortunately not possible to fully

Table 1
In vivo observations for gastric coagulation behaviour of milk in humans and animals.

Factor affecting coagulation	Experiment	Observation	Reference
Milk source			
Cow breeds and dairy herd	Human (infants)	Infants fed with milk with low curd tension experienced less difficulty in digestion and less occurrence of regurgitation and vomit.	Hill (1931)
Milk composition			
Fat content	Human (infants)	Consumption of milk with higher content of fat improved stool frequency and consistency.	Brennemann (1911)
	Human (an adult male with the unique ability to deliver samples of stomach contents at will)	Higher content of cream resulted in the formation of soft curds that are slow to leave the stomach whereas skimmed milk yielded hard curd.	Bergeim et al. (1919)
	Animal (calves)	Milk containing up to six percent of fat formed soft curd milk that leaves the stomach quicker than skim milk.	Espe and Cannon (1935)
Calcium content	Human (infants)	The amount of nitrogen eliminated in the stools of infants was correlated to the calcium content of the food. The increase in the nitrogen content of stools from infants may be due to the presence of protein curds.	Bosworth (1921)
Processing			
Heating	Human (infants)	Consumption of boiled milk improved stool frequency and consistency.	Brennemann (1911)
	Human (adults)	The casein of raw milk formed hard and large coagula, that are not present in boiled milk.	Brennemann (1913)
	Human (an adult male subject with the unique ability to deliver samples of stomach contents at will)	Pasteurised milk resulted in smaller curds than the raw whole milk but larger than the boiled whole milk.	Bergeim et al. (1919)
	Animal (puppies)	Boiled and acidified milk formed loose and small curds in the stomachs of puppies, whereas raw and pasteurised milks formed large tough coagula.	Hess, Koch, & Sennewald (1926)
	Human (infants)	The curds in the infants' stomachs after taking soft curd milk were softer than those after taking unboiled milk, about equal in size to those of boiled milk, but larger and tougher than those of evaporated or breast milk.	Elias (1932)
	Animal (calves)	Soft curd milk left the stomach quicker than normal or hard curd milk.	Mortenson, Espe, & Cannon (1935)
	Animal (mini-pigs)	Slower gastric emptying of raw and pasteurised milk compared with UHT milk. Softer curd from UHT milk compared with raw and pasteurised milk was also observed.	Meisel and Hagemeister (1984)
	Animal (mini-pigs)	Higher levels of amino acid in the blood after feeding mini-pigs with UHT milk.	Kaufmann (1984)
	Animal (rats)	Increased gastric emptying of UHT-treated and sterilised milk compared with pasteurised milk.	Miranda and Pelissier (1987)
	Human (adults)	Compared with pasteurised milk, UHT-treated milk resulted in faster and higher transfer of dietary nitrogen into serum amino acids and protein as well as to urea.	Lacroix et al. (2008)
	Animal (mini-pigs)	Higher levels of blood amino following ingestion of heated compared with unheated skim milk.	Barbé et al. (2013)
	Animal (rats)	UHT milk formed curds with fragmented and crumbled structures, compared with the more cohesive curds formed from unheated or pasteurised milk. UHT milk had faster rates of protein hydrolysis and of the release of fat globules during digestion.	Ye et al. (2019)
Homogenisation	Human (infants)	The digestibility and safety the homogenised milks proved as satisfactory as the control boiled milk.	Wolman, Borowsky, Nicholas, & Spur (1942)

characterise differences between soft curd milk and hard curd milk, but the physiological relevance of casein coagulation was clearly highlighted through the aforementioned work. Likewise, this was also clear from aforementioned work highlighting the importance of casein coagulation in determining the kinetics of postprandial aminoacidaemia. Together, a picture arises suggesting that gastric casein coagulation is important in controlling the kinetics of aminoacidaemia, but also that excessive casein coagulation, leading to the formation of excessively strong curds, should be also avoided, particularly in groups of the population with digestive disorders or discomfort. This, in turn, has led to a large body of research investigating the in vitro coagulation behaviour of milk proteins and the effects of compositional, environmental and processing factors thereon, as discussed later in section 4. Particularly by combining in vitro and in vivo observations on this topic with mechanistic insights for milk protein coagulation under different circumstances (e.g., during the manufacture of cheese or yoghurt) notable advances have been gained, which are discussed in the following sections.

3. Milk proteins and coagulation behaviour in milk

Three classes of protein are distinguished in milk, i.e., the caseins, the whey proteins and the milk fat globule membrane (MFGM) proteins. The latter class represent only a small portion of total milk protein (typically 1–2%), whereas caseins and whey proteins dominate the milk proteins quantitatively. In milk from most major dairying mammals, e.g., cows, goats, sheep and buffalo, caseins and whey proteins occur at a ratio of approximately 4:1, but in human milk this ratio is approximately 2:3.

3.1. Caseins

In the milk of most species, caseins constitute a class of 4 gene products, i.e., α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein (Huppertz, 2013); an exception is human milk, where α_{S2}-casein has, to date, not been discovered (Zhu & Dingess, 2019). Ratios between different caseins vary between species, with primarily the level of α_{S1}-casein being quite variable between and within species. In bovine milk, α_{S1}-casein represents approximately 35% of total casein (Huppertz, 2013), whereas in human milk it represents <10% of total casein (Zhu & Dingess, 2019) and in caprine milk, levels varying between 0 and 40% of total casein have been reported (Devold et al., 2011; Moatsou, Vamvakaki, Molle, Anifantakis, & Leonil, 2006; Pierre, Michel, & Le Graet, 1995).

Unique to all caseins is the lack of a strongly defined secondary or tertiary structure, as well as the fact that all caseins have been subjected to post-translational modification (PTM) (Bijl, Holland, & Boland, 2020; Huppertz, 2013). Glycosylation has been observed in the C-terminal part of κ-casein, with variation observed both between and within species in terms of the number of glycosylated residues and the glycans attached. Furthermore, all caseins contain phosphorylated Ser residues. The level varies strongly between caseins and species (Bijl et al., 2020; Huppertz, 2013). The phosphorylation of the caseins links strongly to that of a key biological functionality of this protein class, i.e., to act as a carrier of bioavailable calcium, phosphate and other minerals from the mother to the neonate. This mineralisation of caseins in the mammary gland leads to the formation of casein micelles, i.e., association colloids which may be considered as natural carriers of minerals (Farrell, Malin, Brown, & Qi, 2006; Huppertz et al., 2017). In addition, the existence of casein in the form of casein micelles, and their controlled destabilisation, are also the basis of the production of e.g., cheese and yoghurt from milk (Walstra, Wouters, & Geurts, 2006), as well as the distinction between fast (whey protein) and slow (casein) dietary protein on digestion (Boirie et al., 1997).

In colloidal terms, casein micelles are best described as sterically-stabilised highly-hydrated association colloids (De Kruif, Huppertz, Urban, & Petukhov, 2012). Taking the bovine casein micelle as an example, an average casein micelle diameter of 150–200 nm is typically reported for bulk milk, with a rather wide size distribution. However, if milk from individual cows is considered, larger variation in particle size between but much narrower size distributions per cow are observed (De Kruif & Huppertz, 2012). On average, the casein micelle consists of approximately 75% water, whereas approximately 93–95% of dry matter consists of protein and the remainder of minerals, most notably calcium and phosphate, but also smaller amounts of magnesium, citrate and other minerals (De Kruif & Holt, 2003; Huppertz et al., 2017; McMahon & Oommen, 2013).

Although some of the minerals may be bound directly to amino acid residues on the caseins (Bijl, Huppertz, van Valenberg, & Holt, 2019), most of the calcium and phosphate are found in so-called calcium phosphate nanoclusters (Bijl et al., 2019; Holt, Carver, Ecroyd, & Thorn, 2013), consisting primarily of amorphous calcium phosphate and having a diameter of 4–5 nm (De Kruif et al., 2012; Holt, De Kruif, Tuinier, & Timmins, 2003). A bovine casein micelle contains several hundred of these nanoclusters (De Kruif et al., 2012; Holt et al., 2003). Centres of phosphorylation (i.e., at least 3 SerP residues in close proximity), which are present on all caseins except κ-casein, can interact with the surface of the nanoclusters and act as growth inhibitors, this preventing uncontrolled formation of (amorphous) calcium phosphate precipitates. Typically, approximately 50 centres of phosphorylation are present on the surface of a nanocluster (De Kruif & Holt, 2003). Caseins also interact via collective weak cohesive interactions (including van der Waals interactions, hydrogen bonding, hydrophobic interactions and electrostatic interactions) into primary casein particles (PCP) prior to nanocluster formation and PCPs consisting of α_{S1}-, α_{S2}- and β-casein can interact with 2 nanoclusters, a network of PCPs linked by calcium phosphate nanoclusters arises to form the core structure of the casein micelles (Huppertz et al., 2017).

While κ-casein is rarely found in the core of the casein micelle, it plays a key role on the surface of the casein micelle, where it provides steric stabilisation to the casein micelle (Dalgleish & Corredig, 2012; Holt & Horne, 1996). While the N-terminal part of κ-casein is attached to PCPs (also containing α_{S1}- and β-casein) that in turn are associated with calcium phosphate nanoclusters, the C-terminal part of κ-casein protrudes from the surface as a so-called hairy layer, providing the micelle with steric stabilisation. This C-terminal part of κ-casein is strongly negatively charged due to the presence of negatively charged amino acid residues (Glu, Asp) and the virtual absence of positively charged residues (Lys, Arg, His). This net-negative charge results in solvency of the C-terminal part of κ-casein (Dalgleish & Corredig, 2012; Holt & Horne, 1996), which is further improved by the fact that all glycosylation of κ-casein is also found in this region (Bijl et al., 2020; Huppertz, 2013).

Despite the notable net-negative charge provided by the hairy layer of κ-casein protruding from the micelle surface, casein micelle stability is still considered to be largely steric rather than electrostatic (De Kruif, 1999). Increasing ionic strength through the addition of, e.g., NaCl, and thereby reducing the range over which electrostatic repulsion occurs, does not induce aggregation of the casein micelles (Huppertz & Fox, 2006). However, if either the height or the density of the κ-casein brush on the micelle surface is reduced, aggregation of casein micelles can occur (De Kruif, 1999). Key routes for achieving this are the removal or collapse of the κ-casein brush on the micelle surface. Collapse of the κ-casein brush occurs due to a loss of solvency, e.g., due to

neutralisation of the net-negative charge as a result of acidification. This process forms the basis of yoghurt preparation from milk, as well as for classical separation of casein and whey protein from skimmed milk by precipitation of the former at pH 4.6. Loss of solvency can also occur due to changes in solvent quality, e.g., as a result of the addition of ethanol to milk (De Kruif, 1999).

The prime example of the removal of the stabilising κ-casein brush from the micelle surface is the enzymatic coagulation of milk as the first step in cheese-making. This process is carried out by the addition of rennet to milk; traditionally, calf rennet has been used, which contains a mixture of chymosin and pepsin. These proteases hydrolyse κ-casein at the Phe_{105}—Met_{106} position, leading to the release of the C-terminal casein macropeptide (CMP) and a loss of stabilising hairs on the surface of the casein micelles. Once sufficient hairs have been removed and bare patches on the micelle surface have become available for interaction, aggregation of para-casein micelles starts, eventually forming a coagulum (Corredig & Salvatore, 2016). Enzymatic coagulation properties of milk are notably affected by many properties, including processing conditions to which the milk is subjected. In addition, a large variation exists between raw milk from different cows, whereby milk samples from some cows are even classified as poorly-coagulating or non-coagulating (Bittante, Penasa, & Cecchinato, 2012).

The area of poorly- or non-coagulating milk has been an active area of research over the past two decades and various factors have been identified and were reviewed by Bittante et al. (2012). Higher proportions of non- or poorly-coagulating milk have been observed for the milk from Holstein-Friesians and some Scandinavian breeds than for, e.g., Brown Swiss and Simmental. Furthermore, protein content and protein composition, particularly casein composition, are important determinants of coagulation properties (Bittante et al., 2012). For casein composition, both the ratio of individual caseins as well as the specific genetic variants of each casein and their degree of post-translational modification have been shown to explain part of the variability in enzymatic coagulation properties of milk (Bittante et al., 2012; Nilsson et al., 2019, 2020). In addition to the protein fraction of milk, the minerals in milk also have a strong effect on enzymatic coagulation of milk. The role of soluble minerals, particularly calcium ions, on enzymatic coagulation is well established; Malacarne et al. (2014) also showed that ratio of micellar calcium and micellar phosphate to casein differed between milk samples with different coagulation properties. Milk samples with a higher mineralisation of the casein fraction showed quicker and stronger coagulation than milk samples with a lower degree of casein mineralisation. Such findings are in line with previous studies by Shalabi and Fox (1982), who showed that increasing micellar calcium phosphate content improved rennet coagulation of milk and reducing it impaired rennet coagulation properties (Shalabi & Fox, 1982).

3.2. Whey proteins

With the exception of human milk (Zhu & Dingess, 2019), the whey proteins represent a smaller fraction of total protein than the caseins. Contrary to the casein fraction, consisting of 3—4 rather closely related gene products, the whey protein fraction is much more diverse, containing a large number of proteins varying in size and biological function. By concentration, the main whey proteins in milk of most mammalian species are β-lactoglobulin (β-LG; which is not present in human milk and camel milk), α-lactalbumin (α-LA), blood serum albumin (BSA) and the immunoglobulins (IGs) (Walstra et al., 2006). Together, these represent >75% of total whey protein in the milk of most species. Despite their variation in, e.g., molecular mass by almost 2 orders of magnitude (14—900 kDa), these whey proteins do have certain aspects in common. Structurally, they all have well defined secondary and tertiary structure, with an important role of intermolecular disulphide bonds in the structure (Boland, 2011; Edwards & Jameson, 2020; Farrell et al., 2004). The structure, however, is prone to heat-induced unfolding and all these proteins have a denaturation temperature <80 °C. Following heat-induced unfolding, the whey proteins can aggregate, with covalent linkages being formed through intermolecular disulphide bonds (Anema, 2020). The free thiol group in β-Lg is crucial in this process to initiate sulfhydryl-disulphide-interchange reactions. Interactions may be with other whey proteins, but also with κ-casein, which contains intermolecular disulphide bonds (Anema, 2020). The heat-induced denaturation has large effects on whey protein functionality, also during gastric digestion, which will be outlined later.

Unlike casein, whey proteins in their native state are hardly prone to coagulation as a result of pH; even at their iso-electric pH (e.g., ~5.3 for β-Lg, ~4.2 for α-La) the proteins remain in solution at temperatures < 50 °C. However, denatured whey proteins are susceptible to (further) aggregation and precipitation in this pH range. Native β-Lg shows no susceptibility to pepsin-induced hydrolysis (Peram, Loveday, Ye, & Singh, 2013) whereas some native α-La may be hydrolysed by pepsin (Miranda, Hazé, Scanff, & Pélissier, 1989). Following heat-induced denaturation, these proteins can show increased susceptibility to pepsin-induced hydrolysis (Peram et al., 2013).

3.3. Milk fat globule membrane proteins

Like caseins, the MFGM proteins are also important in governing the colloidal stability of natural delivery systems in milk, i.e., the milk fat globules. The milk fat globules are particles consisting of a core of neutral glycerides (mainly triglycerides) surrounded by a trilayer membrane containing polar lipids (mainly phospholipids) and proteins. Proteins in the MFGM have important biological functions (for review see Dewettinck et al., 2008). In addition to their biological function, the MFGM proteins also have an important role in providing colloidal stability to the milk fat globules (Jukkola & Rojas, 2017; Singh, 2006; Singh & Gallier, 2017) and a net-negative charge (Dalgleish, 1984). Stabilisation is achieved primarily through steric repulsion as a result of MFGM proteins protruding from the surface into the milk serum (Walstra, 1995). This, in essence, is based on comparable principles as the steric stabilisation of casein micelles by the C-terminal of κ-casein protruding from the interface.

The removal of this steric stabilisation can lead to aggregation of milk fat globules; this is observed after addition of the proteolytic extract papain to milk (Shimizu et al., 1980). However, addition of trypsin or chymotrypsin did not cause coagulation of fat globules in milk (Shimizu et al., 1980), whereas they are able to coagulate casein micelles in milk (Ilany & Netzer, 1969). Whereas steric stabilisation of casein micelles is chiefly achieved by a single protein (Holt & Horne, 1996), the milk fat globules have a collection of proteins with parts protruding from the interface into solution, e.g., butyrophilin, mucins, CD36 and PAS6/7 (Jukkola & Rojas, 2017; Singh, 2006; Singh & Gallier, 2017). The combination of proteins on the interface, with different amino acid composition and PTM, results in different susceptibility to hydrolysis and coagulation. As other proteins retain solubility, no clearly coagulation of milk fat globules may be found, unless a broad-spectrum protease extract like papain is added (Shimizu et al., 1980). The proteins in the MFGM are susceptible to enzymatic hydrolysis by pepsin under (simulated) gastric conditions. As a result, stabilisation of milk fat globules is reduced and coagulation of fat globules can occur under (simulated) gastric conditions, as is discussed later in section 4.2.

4. Gastric coagulating behaviour of milk

4.1. Studying gastric coagulation of milk

For studying gastric coagulation behaviour, both in vitro and in vivo measurements may be considered. For in vitro digestion, a wide variety of systems are used, which can be classified as either static, semi-dynamic or dynamic (Bohn et al., 2018; Dupont et al., 2019; Dupont & Mackie, 2015; Egger et al., 2019). When comparing data from in vitro and in vivo studies, some care should be taken as in vitro studies may not fully reflect the in vivo conditions. Simple static in vitro systems may focus simply on a single addition and pH adjustment after which digestion or coagulation is monitored. In reality, however, there will be a gradual addition of gastric juice, as well as gastric emptying (Bohn et al., 2018; Dupont et al., 2019; Dupont & Mackie, 2015; Egger et al., 2019). This leads to changes in the stomach content, specifically: (i) pH will decrease gradually, (ii) protein content will decrease gradually, (iii) enzyme:substrate ratio will increase gradually.

All factors are known to influence casein coagulation and should be considered in translation of in vitro to in vivo. However, static and semi-dynamic systems can still be extremely valuable in attaining mechanistic insights.

Furthermore, consideration should be given to the specific conditions in the stomach and gastric juice e.g., in terms of pH and enzyme concentration of the gastric secretions. As notable variability is observed throughout stages of life, conditions in digestive systems should reflect the target population of the study (Shani-Levi et al., 2017). For instance, the stomach of an infant is immature and undergoing development, as shown in Fig. 1. The newborn infant's digestive capability is comparatively low, with the pepsin activity functioning at merely 10% of an adult's capacity. Anatomically, the stomach dimensions and capacity increase rapidly during the first year of life. A neonate has a resting stomach volume as small as 15 mL, which grows to around 210 mL in one year. The average protein requirement for an infant is estimated to be around 1.12 g kg^{-1} day-1 at the age of 6 months (Garlick, 2006). To meet the high nutritional requirement with a limited stomach capacity, frequent feedings are necessary. An abnormal gastric emptying is posing pressure to this delicate system, which could potentially lead to disrupted satiety regulation and food intake, as well as digestive disturbance such as vomiting and regurgitation (Doan, 1938; Goyal, Guo, & Mashimo, 2019).

When reflecting on gastric coagulation of milk, potential causes for deviation are found in the manner of addition of gastric juice. Furthermore, it is imperative to keep in mind that the stomach is by no means static. Muscle contractions will generate mechanical forces and fluid motions, affect the coagulation and formulation of coagula. Fluid dynamics modelling of gastric motility indicates that particularly strong flow fields can occur in the antropyloric region, whereas this is far more limited in the fundus and corpus (Ferrua & Singh, 2010; Kozu et al., 2010). The strength of flow fields decreases with increasing viscosity of stomach content (Ferrua & Singh, 2010). Depending on the predominant mode of aggregation of particles, i.e., orthokinetic or perikinetic, aggregation will either be enhanced or reduced by the flow fields encountered. For particles prone to predominant orthokinetic aggregation, coagulation may be enhanced compared with the case under quiescent conditions, whereas for perikinetic aggregation, the disruptive effect of the forces on structures formed will predominate (Kim & Kramer, 2006; Vanapalli, Coupland, Friberg, & Larsson, 2004). For coagula already formed, flow fields may result in disruption.

The size and strength of structures present in the gastric digesta is important for gastric emptying. For liquids devoid of particulate matter, gastric emptying rate is determined primarily by gastric volume and energy density and osmolarity of the liquid (Khoshoo & Brown, 2002). However, when solid foods or liquid foods containing particulate matter are ingested, particle need to be sufficiently small (<1–2 mm) for before they can be emptied from the stomach (Kong & Singh, 2008). This may require physical degradation of material in the stomach. For milk, no particulate matter is present on ingestion, but coagulation occurs under gastric conditions, as outlined further in detail below.

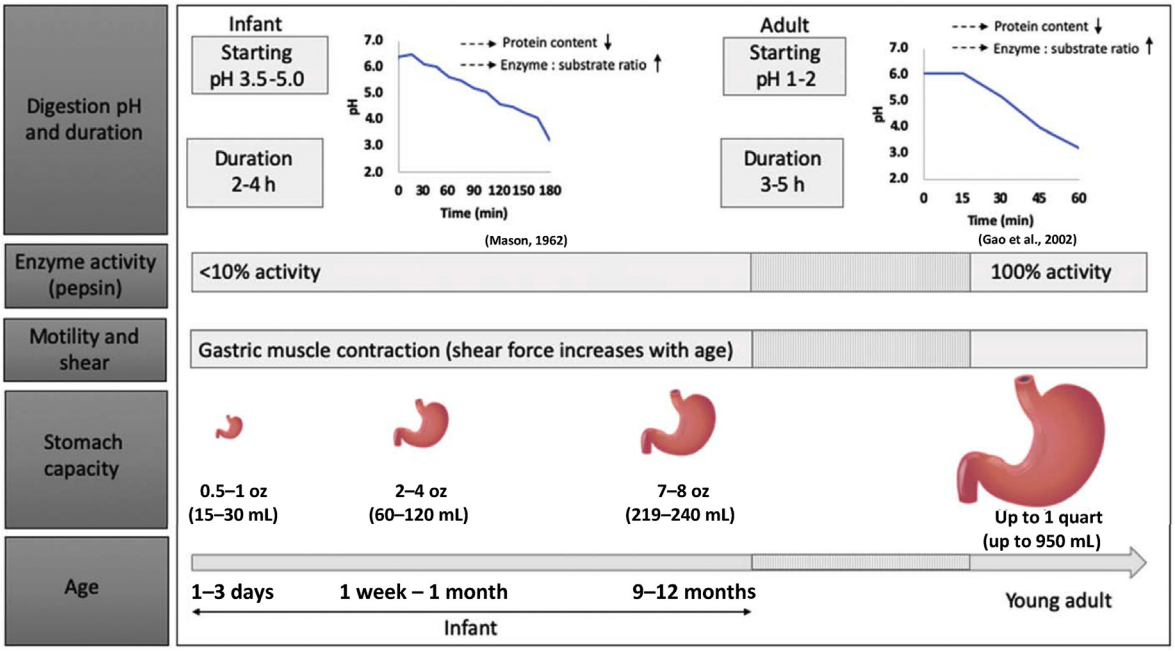

Fig. 1. Developmental changes in the stomach of a new-born. An infant stomach undergoes rapid anatomical and physiological development during the first year of life in terms of gastric secretion, enzyme activity, motility/shear, and stomach capacity. After milk consumption, the pH dynamics of stomach contents differ between infants (data redrawn from Mason, 1962) and adults (data redrawn from Gao et al., 2002).

4.2. Gastric coagulation behaviour of raw milk

When considering gastric coagulation of milk, many aspects observed broadly resemble those observed during enzymatic coagulation of milk during cheese making and acid coagulation of milk during yoghurt making. When milk is consumed, it enters the stomach where a small amount of gastric fluid is present. Although this gastric fluid is acidic (i.e., as shown in Fig. 1, pH 1–2 for adults, pH 3.5–5.0 for infants) it has limited buffering capacity and volumes present in a fasting stomach are typically small compared with the volume of milk ingested. Hence, pH of the stomach content increases strongly and can easily reach values > 6.0 when e.g., a 300 mL portion of milk is consumed by an adult (Gao, Mitsui, Fujiki, Ishiguro, & Kondo, 2002) or when infants are fed on human milk or infant formula (Mason, 1962).

Both in vivo and in vitro studies have shown that for unheated or mildly heated milk, coagulation occurs rather quickly (e.g., within <30 min) after ingestion (Bergeim, Evvard, Rehfuss, & Hawk, 1919; Ye, Cui, Dalgleish, & Singh, 2016a). The pH of the stomach contents is still rather high (often pH > 5.5) at this point (Fig. 1), suggesting that the destabilisation reactions leading to coagulation are predominantly enzyme-induced rather than acid-induced. This enzyme-induced coagulation, initiated by the pepsin-induced hydrolysis of κ-casein, leading to the release of CMP and para-casein micelles, as seen using SDS-PAGE by Ye et al. (2016a). Whey proteins in their native state are not susceptible to coagulation gastric coagulation and thus not included in the casein curd formed (Ye et al., 2016a).

Milk fat globules present in raw milk have also been shown to become entrapped in the curd particles and are typically seen evenly distributed throughout the protein coagulum formed (Ye et al., 2016b, 2017). This seems in a manner similar to occurring during cheese-making, where para-casein matrix entraps the fat globules (Guinee & McSweeney, 2006; Ong, Dagastine, Kentish, & Gras, 2011). Over time, however, coalescence of fat globules is observed microscopically in gastric curds (Ye et al., 2016b, 2017). This coalescence can be promoted by (simulated) gastric motility during in vitro digestion as well as by contraction of the casein matrix, as also observed during cheese-making (Lopez, Camier, & Gassi, 2007; Richoux et al., 2008). Furthermore, it should also be noted that some of the proteins on the MFGM are hydrolysed by pepsin (Gallier et al., 2013; Kobylka & Carraway, 1973; Le et al., 2012; Vanderghem et al., 2011; Ye et al., 2011), which will reduce colloidal stability of the milk fat globules and make them more susceptible to coagulation. This was also exemplified in cream by Gallier et al. (2013) who observed increases in fat globule size during in vivo gastric digestion of cream in rats. Due to the fact that the (low) levels of casein present in cream are insufficient to form a protein matrix in which the fat globules in become embedded, increases in fat globule size could be directly monitored. Aggregation of milk protein-stabilised emulsions in the stomach can also lead to creaming in the stomach, as shown by MRI studies (Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013). Such creaming is probably also the reason for delayed gastric emptying of curds from whole milk compared with skim milk, as observed in vivo (Bergeim et al., 1919) and in vitro (Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019).

Just as for rennet-induced coagulation, where notable differences in coagulation behaviour are observed between milk from individual cows (Bittante et al., 2012; Nilsson et al., 2019, 2020; Poulsen et al., 2013; Poulsen, Glantz, Rosengaard, Paulsson, & Larsen, 2017), notable differences between raw milk samples from individual cows are also found in gastric coagulation.

An extensive body of work on this topic was carried out in the first half of the 20th Century, where the coagulation behaviour of milk was investigated in relation to ease of digestion. Distinction was made between so-called soft-curd milk and hard-curd milk, whereby the former was favoured from a digestive perspective (see Section 2). Milk samples could be classified as soft-curd or hard-curd based on the firmness of the gel formed after incubating milk with pepsin for a defined time and measuring the firmness (or tension) of the formed curd (Hill, 1928, 1931). A notable difference could be observed between milk from Holstein cows versus Jersey cows, with the milk from the latter giving considerably stronger curds (Hill, 1928, 1931). Part of this difference may be attributed to differences in gross composition of the milk, with particularly the higher protein content of Jersey milk contributing to stronger curds.

However, results from Hill (1928, 1931) in terms of coagulation behaviour do not seem to be only attributable to variation in protein content and other factors, like for renneting, also contribute. One factor is protein:fat ratio, as skimming of milk prior to determination of curd firmness led to increases in curd firmness, indicating a disruptive effect of the presence of fat globules in milk (Bergeim et al., 1919). This, again, draws parallels to rennet-induced coagulation of milk, where the presence of fat can also reduce curd firmness at temperatures >30 °C (Michalski, Cariou, Michel, & Garnier, 2002; Zhou & Mulvaney, 1998), due to the fat globules acting as inactive fillers in the para-casein matrix. Variation in gastric coagulation behaviour between different milk samples may also be related to variation in the minerals associated with the casein fraction, as outlined previously for rennet-induced coagulation (Malacarne et al., 2014). Huppertz and Lambers (2020) recently showed that variations in casein mineralisation strongly affects the coagulation behaviour of casein micelles in model infant formula systems. Samples with lower casein mineralisation were found to yield smaller and weaker gastric curd particles. This is in line with findings by Weisberg, Johnson, and Mccollum (1933) that soft curd milk contained less calcium and phosphorus than hard curd milk.

As outlined above, coagulation behaviour of milk in the stomach appears to occur rather soon after ingestion and appears to be an enzymatic coagulation process initiated by the hydrolysis of κ-casein. During further (simulated) gastric digestion of raw whole milk, a number of changes are observed in the coagulum; i.e., coagulum weight (both wet weight and dry matter) decreases with progressive digestion, but the wet weight decreases more rapidly (Ye et al., 2016a,b). This suggests both losses of dry matter but also additional expulsion of water. The ratio of fat:fat-free dry matter in the coagulum, however remains rather constant (Ye et al., 2016b), indicating that losses in dry matter are proportional for fat and fat-free dry matter. Microscopically, the network of the coagulum also appeared to become much denser, and coalescence of fat globules was seen to occur (Ye et al., 2016b; 2016a). In general, such processes bare resemblance to syneresis processes observed during cheese-making (Dejmek & Walstra, 2004).

Various factors should be considered in relation to changes in the coagulum during prolonged digestion. Further enzymatic degradation of caseins by pepsin will occur, with all other caseins also degraded during prolonged incubation (Ye et al., 2016a). This process of proteolysis, however, may be hindered by the contraction of the coagulum, limiting diffusion and accessibility to substrate (Ye et al., 2016a). While some of the (poly)peptides remain associated with the (remnants of the) casein micelles, others are released into the serum phase (Ye et al., 2016a). Furthermore, the decrease in gastric pH can lead to solubilisation of some of the micellar calcium phosphate (Le Graët & Gaucheron, 1999), which can weaken the gel structure for para-casein gels in cheese and related products (Lucey & Fox, 1993). It is likely, however, that this will occur primarily around the surface of the curd particles, as diffusion of acid into the matrix, and transport of solubilised

calcium and phosphate out of the matrix, will be hindered by the coagulum structure and further contraction thereof. Furthermore, the reduction in pH would also bring the conditions closer to the iso-electric pH (pI) of (intact) caseins and thereby be expected to strengthen protein interactions (Huppertz, 2013).

However, it is unclear whether this is also the case for the peptides remaining in the micellar after hydrolysis with pepsin. Para-κ-casein, for instance, has a net-positive charge even at neutral pH and acidification thus does not bring it closer to its pI (Huppertz, 2013). Further study is required to identify casein-derived peptides remaining in the micelle/coagulum and their properties and interactions. In addition to further proteolysis and pH decrease, a factor that can further affect the gastric coagulum is gastric motility and syneresis. Post-coagulation, gastric motility will likely lead to breakdown of structures (Ye et al., 2016b). However, limited gastric motility can also lead to syneresis of the coagulum, resulting in serum release and making coagulum particles more compact, as also occurs on cheese-making (Dejmek & Walstra, 2004). Once coagulum particles become small enough (<1–2 mm), through breakdown or syneresis, they can be emptied through the pylorus.

4.3. Impact of processing on gastric coagulation of milk

In addition to variations between milk from different breeds or individual cows within a breed, processing also has a strong effect on the gastric coagulation of milk. Within this context, two processing steps appear to have the largest effect, i.e., heat treatment and homogenisation. For heat treatment, the effects on gastric coagulation observed are largely related to the heat-induced denaturation of whey proteins. For homogenisation, the effects observed on gastric coagulation are primarily due to the reduction in fat globule size and the change in the composition of interfacial material stabilising the milk fat globules.

4.3.1. Effect of heat treatment

On heat treatment at temperatures > ~70 °C, denaturation of the whey proteins in milk can occur (Anema, 2020). The extent of whey protein denaturation increases with increasing temperature and treatment time, typically reaching values > 90% after, e.g., 10 min at 90 °C or 2 min at 120 °C. As a result of heat-induced denaturation, the whey proteins start aggregating, a process that appears to be governed by the main whey protein, β-lactoglobulin. As a result, various aggregates can be created. These can consist of only whey proteins, but also of whey proteins and caseins, particularly κ-casein. The aggregates containing both caseins and whey proteins can occur in the serum phase of the product, as some κ-casein dissociates from the micelle surface on heat treatment, but also on the surface of the casein micelles (Anema, 2020). Particularly the presence of κ-casein-whey protein aggregates on the surface of the casein micelles strongly affects enzymatic coagulation processes, including gastric coagulation.

As the interaction of whey proteins with κ-casein occurs in the N-terminal part of κ-casein, where both Cys-residues are located, it does not hinder the enzymatic hydrolysis of κ-casein and the release of CMP (Vasbinder, Alting, & De Kruif, 2003). However, since the complexed whey protein remains on the surface of the micelle after CMP is released, the para-casein micelles in this case retain more steric stabilisation then in the case of unheated milk (Guinee et al., 1997; Singh & Waungana, 2001; Vasbinder et al., 2003). Hence, enzymatic coagulation is at the very least hindered, but may also be prevented if heat treatment has resulted in high levels of whey protein denaturation. For cheese-making, this leads to the formation of an insufficiently firm coagulum for further processing (Kelly, Huppertz, & Sheehan, 2008). However, for gastric coagulation, heat treatment and the resultant whey protein denaturation can be used as a way to control gastric coagulation and gastric transit (Mulet-Cabero et al., 2019; Ye et al., 2016a,b, 2017).

The effect of heat treatment of milk on gastric coagulation and digestion were already recognised at the start of the 20th Century, with several reports on the application of heat treatment as a way of creating a 'soft-curd milk' (Brennemann, 1913; Dizikes & Doan, 1942). Since then, the effect of heat treatment on gastric coagulation of milk has been confirmed in several in vitro and in vivo studies.

In vivo, Lacroix et al. (2008) have shown that compared with pasteurised milk, UHT treated milk resulted in faster and higher transfer of dietary N into serum amino acids and protein as well as to urea in humans. The authors suggested that differences were due to enhanced digestive kinetics. This is in line with studies in rats, where increased gastric emptying of UHT-treated and sterilised milk compared with pasteurised milk was observed (Miranda & Pelissier, 1987). Slower gastric emptying of raw and pasteurised milk compared with UHT milk was also observed in mini-pigs (Meisel & Hagemeister, 1984), and the formation of softer curd from UHT milk compared with raw and pasteurised milk was also observed in mini-pigs (Buchheim, 1984; Meisel & Hagemeister, 1984; Pfeil, 1984), as were higher levels of amino acid in the blood after feeding mini-pigs UHT milk (Kaufmann, 1984). Barbé et al. (2013) also showed higher levels of blood amino acids in mini-pigs following ingestion of heated (10 min at 90 °C) compared with unheated reconstituted ultra-low-heat skim milk powder. However, they also suggested an increased retention time of heated compared with unheated milk in the stomach, which appears contradictory to the findings on blood amino acid levels. In rats, both heat treatment of milk at 85 °C for 15 s or 140 °C for 4 s were shown to form softer gastric curds, which emptied quicker from the stomach, compared with raw milk (Ye et al., 2019). Overall, in vivo studies thus indicate that as a result of heat treatment of milk, weaker curds form in the stomach, as a result of which gastric transit and amino acid uptake in the blood are enhanced.

In vitro, heat treatment of milk has also been shown to have notable effects on gastric curd formation. Compared with pasteurised skim milk, the gastric curd from skim milk heated at 90 °C for 20 min was less dense and more porous (Ye et al., 2016a). The looser structure increased casein digestibility, presumably due to increased substrate accessibility for pepsin. The weaker gastric curd from heated milk was also more prone to fracture by (simulated) gastric contractions (Ye et al., 2016a). For whole milk heated at the same conditions, similar effects of heat treatment were observed (Ye et al., 2016b). Also, weaker, more open curd particles were observed wherein the caseins were hydrolysed more readily by pepsin. A comparison between raw, pasteurised and UHT-treated (3 s at 140 °C) (unhomogenised and homogenised) whole milk showed similar effects (Mulet-Cabero et al., 2019). Particularly gastric curds from UHT treated milk were softer and more fragments than those from raw milk. In this case, however, no strong differences in proteolysis, as studied by SDS-PAGE, were observed between digesta from raw and heated milk samples (Mulet-Cabero et al., 2019).

Overall, effects of heat treatment on gastric coagulation and digestion of milk from both in vitro and vivo studies in humans, rats and mini-pigs show a rather consistent picture; i.e., gastric curds formed from heated milk are softer and more porous than those from unheated milk. As a result, the curds are more susceptible to mechanical disruption and the caseins in the curds are more susceptible to proteolysis. While heat treatment has been proposed for making so-called soft-curd milk, it is important to realise that this may also affect protein digestion negatively. Protein glycation, which can occur as a result of heat treatment, can impact protein

digestibility (van Lieshout et al., 2020; Zenker, van Lieshout, van Gool, Bragt, & Hettinga, 2020) and also the appearance of lysine in the blood (Nyakayiru et al., 2020).

4.3.2. Effect of homogenisation

Homogenisation is typically applied to reduce the size of emulsion droplets and thereby prevent creaming in liquid products. In addition to reducing the size of the emulsion droplets, homogenisation can also affect the protein composition on the emulsion droplet interface. On homogenisation of milk, there is insufficient MFGM material present and milk proteins become adsorbed onto the emulsion droplet interface (Walstra et al., 2006). In other products, such as infant formula and medical nutrition products, where bulk fats and oils are often used as ingredients, proteins also form emulsion droplet interface (Masum, Chandrapala, Huppertz, Adhikari, & Zisu, 2020).

The presence of milk proteins in the interface has large impact on the behaviour of the emulsion droplets under gastric conditions. When >40% of the interface of fat globules is formed by milk proteins (with casein dominating), milk fat globules become prone to enzymatic and acid-induced coagulation (Michalski et al., 2002) and can interact with the para-casein matrix. Although this would be expected to lead to a stronger coagulum, in vitro studies comparing that gastric digestion unhomogenised and homogenised whole milk indicate that the curds formed from the latter are less cohesive and more brittle than curds formed from the former (Mulet-Cabero et al., 2019; Ye et al., 2017). Fat was also release more easily from the gastric curd formed from homogenised milk compared with unhomogenised milk (Mulet-Cabero et al., 2019; Ye et al., 2017). In vivo, homogenisation has been shown to also lead to softer and smaller curds (Anthony, 1936) and improve digestibility (Dizikes & Doan, 1942) compared with unhomogenised milk.

The weaker gastric curd from homogenised milk compared with unhomogenised milk are in agreement with observations on cheese-making from milk, where curds from homogenised milk have also been reported to be less syneresing and more brittle compared with those from unhomogenised milk (Kelly et al., 2008). Kelly et al. (2008) related this to the fact that due to presence of a much higher number of fat globules in homogenised milk compared with unhomogenised milk, the inter-particle difference in milk is considerably smaller. As fat globules are also homogenously distributed in curds prepared from these milks, the inter-particle difference is also considerably smaller. The presence of these fat globules at much smaller length scales hinders the fusion and syneresis of the casein matrix over larger length scales, thus leading to increased moisture retention and more brittle curds (Kelly et al., 2008). While this may be perceived negative in cheese production, it may be positive for gastric digestion, as overly firm gastric curds can be avoided like this.

5. Conclusions and future perspectives

When considering the gastric coagulation of the proteins in milk, it is clear that a multifaceted perspective, including biochemistry, colloid science, physical chemistry and human physiology can provide a self-consistent picture. Many recent in vitro studies on gastric behaviour of milk proteins, as significant advances in the general understanding of milk protein structure and interactions can be applied to provide mechanistic perspectives to (sometimes Century-) old findings on the influence of milk type and milk processing on milk protein digestion. Gastric coagulation regulates gastric emptying, making the process essential in efficient supply and utilisation of protein in the human body. Variations between milk samples, but also as a result of processing strongly affect the coagulation behaviour of milk under gastric conditions and thereby further breakdown of the coagulum and gastric emptying. This allows control over and tailoring of the process via compositional and process variations for products with distinct digestive properties.

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