

# Protein Digestion of Baby Foods: Study Approaches and Implications for Infant Health

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Protein digestion is critical for infants. Dissimilarities between infants and adults in food intake and digestive physiology lead to distinct patterns of proteolysis between individuals. However, such differences are not well represented in many studies on protein digestion of baby foods. The complex biological structures of baby foods and the physiology of the infant digestive system are key factors affecting proteolysis during the first two years of life. Well-controlled in vitro studies have demonstrated that varying digestion conditions alter the specificity, rate, and extent of proteolysis of baby foods. Nonetheless, these models do not completely replicate in vivo proteolysis or the complex biogeography of the gastrointestinal tract. Animal and clinical studies have revealed the fate of dietary proteins along the digestive tract and the overall health impact on subjects. Building comprehensive and annotated datasets from human infants will require innovative and standardized measurement. Now, more systematic evaluations of digestion are emerging to advance the knowledge and its translation as food design for effective diet and health management in infants.

## 1. Introduction

Protein intake early in life is essential for infant health, affecting growth, body composition, neurodevelopment, appetite and hormonal regulation.<sup>[1]</sup> Problems with protein digestion cause short-term and long-term adverse consequences, such as pain, diarrhea, intolerance, allergy, malabsorption, and constipation.<sup>[2]</sup> After ingestion, proteins in baby foods undergo proteolysis within the infant digestive tract before being absorbed or excreted. During this process, some of the proteins are partially digested. These proteins and their peptide fragments can interact with other dietary molecules and cellular components in infants, exerting beneficial functions including nutrient delivery and

antimicrobial activity,<sup>[3]</sup> or leading to deleterious effects such as inflammation and allergic responses.<sup>[4,5]</sup> Therefore, understanding how dietary proteins are chemically and biologically processed during infant digestion has profound implications for food and health.

Due to their immature state, infants require baby foods that are suited to their development. Properties of baby foods are critical for protein breakdown during digestion. Based on physical and chemical characteristics, baby foods can be classified into three categories: breast milk, human milk substitute, and complementary food. Breast milk has evolved under selective pressure to be the initial complete diet for newborns, providing nutrition and protection. As such, exclusive breastfeeding for 6 months followed by continued breastfeeding up to 1 or 2 years is recommended.<sup>[6,7]</sup> Human milk

substitute as infant formula is given to infants when breast milk is unavailable.<sup>[8]</sup> Despite substantial efforts invested to make human milk substitute similar to breast milk, the chemical and structural, much less the biological properties of breast milk, are still impossible to accurately reconstruct.<sup>[7,9]</sup> Complementary foods are nutrient-containing foods other than breast milk that are introduced to infants generally between 6 and 24 months of age, as a complement to breast milk or breast milk substitute.<sup>[10]</sup> The composition, structure, and texture of complementary foods, such as cereal and meat puree, are distinct from breast milk in many ways,<sup>[8,11]</sup> resulting in different patterns of protein digestion.

Besides varying food intake, physiological dissimilarity between infants and adults contributes to divergent patterns of food digestion. However, conditions of infant digestion have not been well represented in many in vitro studies. The growing concern about building in vitro models based on specific infant physiology has been highlighted in recent reviews. Bourlieu et al.<sup>[12]</sup> reviewed studies that characterized gastroduodenal conditions in full-term and preterm infants from birth to six months. Poquet and Wooster<sup>[13]</sup> analyzed characteristics of infant physiology that are related to lipid digestion. In terms of protein digestion, the immaturity of the infant digestive system affects the efficacy of protein digestion and absorption, leading to unique issues in this population. For example, cow's milk allergy primarily affects infants under two years old, and its incidence declines among children older than three years old.<sup>[14]</sup>

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Regarding the relationship of diet to infant health, infant-specific research models are needed to understand protein digestion in more detail. This review will identify key features that affect the hydrolysis of proteins in baby foods, highlight approaches that are adapted for conditions of infant protein digestion, and discuss challenges and directions of future work in this field.

## 2. Properties of Baby Foods that Affect Protein Digestibility

### 2.1. Protein Molecular Structure

The sequence of amino acids in a protein is an intrinsic feature that affects its digestibility. For example, gluten, which is present in baby foods sourced from wheat and rye, contains gliadin peptides that are resistant to gastrointestinal breakdown. The abundance and location of proline residues in gliadin confers its resistance to gastric and intestinal proteases.<sup>[15]</sup> Moreover, the folded structure of a protein influences its digestibility.<sup>[16]</sup> Hydrogen bonds stabilize proteins and can create a compactly folded tertiary structure, making it difficult for enzymes to access cleavable peptide bonds. Post-translational modifications might block proteases from binding and inhibit the enzymatic activity; thus, dephosphorylation of bovine milk protein concentrate increases protein digestibility in an infant in vitro model.<sup>[17]</sup> Nevertheless, effect of modifications on digestibility is negligible in some cases, for example, glycosylation of codfish parvalbumin.<sup>[18]</sup> Disulfide bonds or non-disulfide crosslinking can adversely affect protein digestibility, and breaking disulfide bonds of proteins in soybean and sorghum increases in vitro digestibility.<sup>[19,20]</sup>

Resistance to digestion is a characteristic of food allergens.<sup>[21]</sup> This molecular property allows the intact proteins or their large fragments containing epitopes to reach the gut and trigger adverse immune responses in high risk populations.<sup>[4,22]</sup> Cow's milk protein is the most common protein source for infant formulas, and cow's milk protein allergy is the most prevalent food allergy in early childhood.<sup>[23]</sup> Partially or extensively hydrolyzed infant formulas have been developed to reduce allergenicity and recommended for high risk infants who are not exclusively breast-fed.<sup>[24–26]</sup> However, systematic reviews and meta-analyses on clinical studies have shown contradictory findings on the efficacy of hydrolyzed formulas in preventing allergic diseases in infants.<sup>[24–28]</sup> Molecular structure of proteins and peptides is important for the digestibility and allergenicity, but it was not well characterized in infant formulas and its changes were not annotated along infants' digestive tract, which could be a confounding variable in epidemiological analyses.

### 2.2. Food Composition

Baby foods typically consist of multiple components besides proteins, and non-protein components may have chemical interactions with the proteins. For example, cereal grains like sorghum contain polyphenols, polysaccharides and phytic acid that can crosslink with or bind to proteins, thus decreasing their digestibility.<sup>[16]</sup> Reducing sugars react with proteins in food dur-

ing thermal processing through non-enzymatic browning, the Maillard reaction, modifying the extent of proteolysis and the bioavailability of proteins.<sup>[29,30]</sup> In addition to interacting with proteins of the food, naturally occurring inhibitors from legumes and bovine colostrum can inhibit human trypsin without adequate processing.<sup>[31]</sup>

Baby foods often go through processing procedures such as spray-drying or extensive cooking that are necessary for safety. However, processing operations can cause unintended consequences on proteins in baby foods, changing their structures and interactions. Thermal processing can reduce antinutrients, like tannins and phytic acid in legumes, and improve protein digestibility,<sup>[32]</sup> whereas denaturation, crosslinking between amino acid residues, and the Maillard reaction decrease protein digestibility. Infant formula is particularly susceptible to the Maillard reaction due to the high content of the reducing sugar lactose, which reacts with lysine and reduces its availability.<sup>[30,33]</sup> Lysine is an essential amino acid critical for infant growth; therefore, the lysine blockage has been raised as a nutritional concern.<sup>[33]</sup> This concern has led to advancement in recommendations for formula composition (6.7 g of available lysine per 100 g protein), analytical methods to measure lysine bioavailability, and processing conditions to optimize the quality of infant formulas.<sup>[30,33,34]</sup> Steam-cooking of meat-based baby foods seems to hinder in vitro proteolysis, although it reduces allergic responses in children with atopic dermatitis compared to raw meat.<sup>[35]</sup>

### 2.3. Food Structure

Baby foods are diverse in their physical structure: the spatial arrangement of carbohydrates, lipids, and proteins varies considerably from sweet potato mash to meat puree.<sup>[36–39]</sup> The structure of baby food affects the rate and extent of its protein digestion, for digestive processes are to convert a structured food into a bioavailable form. A milk emulsion of the native milk fat globule structure has a lower rate of proteolysis during in vitro gastric digestion compared to homogenized or homogenized/pasteurized infant formulas.<sup>[40]</sup> The adsorption of proteins to lipid interfaces in an emulsified food or the protein-lipid interactions can change the accessibility of cleavage sites to digestive enzymes and modify the local flexibility of substrates.<sup>[41]</sup> For grains, such as rice, maize and sorghum, the structure of protein bodies and endosperm cell walls affects the stability of their storage proteins during cooking and digestion.<sup>[42,43]</sup> Since food properties are variables affecting proteolysis, characterization of baby foods is valuable to obtain insights into infant protein digestion.

## 3. Infant Physiology that Relates to Protein Digestion

Digestion is a complex process of mechanical and chemical breakdown of food that involves multiple organs and tissues.<sup>[44]</sup> To reduce its complexity for studies, this process is generally dissected into three physiological phases: oral phase, gastric phase, and intestinal phase.



**Figure 1.** Development of oral behaviors in response to foods.

### 3.1. Oral Phase

During the oral phase, food materials are broken down and lubricated with saliva before being swallowed.<sup>[45]</sup> These processes change the particle size and viscosity of the ingested food, increasing protein accessibility. The oral phase is often neglected when modeling infant digestion because of the relatively short transit time of liquid foods in newborn infants' oral cavity.<sup>[46]</sup> However, significant oral processing of food occurs with teething and neural development of oral movement control. Infants are introduced to semi-solid and solid foods during 6 months to 2 years, and they respond with different chewing and swallowing behaviors to help food breakdown.<sup>[47,48]</sup>

As infants' oral cavity and nervous system develop, the main pattern of their oral movement shifts from suckling to chewing (Figure 1). At the age of 4 to 6 months, infants move the tongue gently back and forth as food enters mouth.<sup>[49]</sup> At 6 months, most infants are able to remove pureed foods by full lip occlusion around the spoon, yet they have difficulty retaining viscous and solid foods.<sup>[47]</sup> From 6 to 10 months, infants can eat foods with tiny lumps without gagging.<sup>[49]</sup> Around 8 months, teeth erupt;<sup>[50]</sup> from 12 to 24 months, initiation of chewing becomes predominant.<sup>[47,48]</sup> In parallel, their eating behavior becomes more efficient, including better lip control, increased tongue mobility, and decreased involvement of lips and cheeks in swallowing.<sup>[47]</sup> Also, both the time of chewing and the number of chewing cycles to process food before swallowing decrease as infants grow.<sup>[51]</sup> One hypothesis for these decreases is that older children, with increased eating efficiency, require less efforts to achieve the same particle size or viscosity of the food after chewing, which may hold constant across ages.<sup>[47]</sup> Unfortunately, the particle size and texture of bolus before swallowing by infants are unknown, since obtaining such information requires subjects to spit out the chewed bolus before swallowing, which is challenging to implement with infants. Though bite force of infants is unknown, data from children indicates a positive correlation between bite force and age. In particular, bite force increases from  $196.0 \pm 96.1$  to  $480.2 \pm 162.4$  N in children from 3–5 to 12–14 years

old, respectively.<sup>[52]</sup> No significant difference exists between pre-pubescent boys and girls.<sup>[52,53]</sup>

Ben-Aryeh et al.<sup>[54]</sup> analyzed whole unstimulated saliva of healthy infants aged 3 days to 12 months, and compared to data from adults. Significant differences of salivary compositions were not found in terms of diet, tooth eruption or gender, but were observed between age groups.

### 3.2. Gastric Phase

Gastric juice contains water, hydrochloric acid, intrinsic factor, enzymes, and mucus. Infants' gastric secretory capacity were studied by Agunod et al.<sup>[55]</sup> and Rodbro et al.<sup>[56]</sup> Their findings show that gastric secretion in infants was below adult level (Table 1a and 1b).

A direct measurement to elucidate effects of gastric acid on protein digestion is gastric pH in response to a meal. Fluctuation in pH leads to conformational changes of enzymes and proteins, affecting enzyme-substrate interactions. Figure 2 shows the change of gastric pH after meal in healthy infants and adults.<sup>[57–63]</sup> While postprandial gastric pH of infants is relatively high compared to adults, it depends on gastric acid secretion as well as meal content. When food is ingested, parietal cells in the gastric glands are stimulated to secrete acid into the stomach lumen.<sup>[44]</sup> This process, regulated by the nervous and endocrine system, is limited by the secretory capacity of infants.<sup>[55,56,64]</sup> Meanwhile, foods like breast milk, with a strong buffering capacity and a slow gastric emptying, can slow down the decrease of gastric pH.<sup>[57,65]</sup> Compared to formula, clear liquid (glucose-electrolyte solution) resulted in a lower gastric pH in preterm infants (Figure 3).<sup>[65]</sup>

The major digestive protease in human stomach is pepsin. Pepsinogen is secreted by gastric chief cells and activated into pepsin via autocatalytic cleavage of an N-terminal peptide when it comes in contact with acid. As an aspartic protease, pepsin is most active in acidic environments around pH 2, and inactivated at pH above 4.5.<sup>[44]</sup> Questions remain whether pepsin

**Table 1a.** Gastric outputs after stimulation with Histalog (1.0 mg/kg body weight) in full-term infants (1–110 days), children (4–9 years), and adults. Adapted from Agunod et al.<sup>[55]</sup>

| Subjects    |       | Body weight (kg) | Volume (mL/hr) | Acid (mEq/hr) | Pepsin (mg/hr) |
|-------------|-------|------------------|----------------|---------------|----------------|
| 1 day       | Mean  | 3.4              | 3.3            | 0.03          | 0.18           |
| (n = 10)    | Range |                  | 0.8–9.3        | 0.01–0.9      | 0–0.53         |
| 3–8 days    | Mean  | 3.3              | 3.7            | 0.06          | 0.21           |
| (n = 7)     | Range |                  | 1.3–4.7        | 0.01–0.12     | 0.11–0.44      |
| 10–11 days  | Mean  | 3.0              | 4.0            | 0.12          | 0.46           |
| (n = 5)     | Range |                  | 2.0–5.4        | 0.11–0.15     | 0.29–0.67      |
| 14–17 days  | Mean  | 3.4              | 6.4            | 0.19          | 0.88           |
| (n = 4)     | Range |                  | 1.5–12.0       | 0.04–0.37     | 0.20–1.46      |
| 25–32 days  | Mean  | 3.9              | 3.1            | 0.08          | 0.32           |
| (n = 3)     | Range |                  | 3.0–3.2        | 0.03–0.14     | 0.21–0.38      |
| 67–110 days | Mean  | 4.9              | 13.4           | 0.47          | 1.34           |
| (n = 4)     | Range |                  | 7.1–19.9       | 0.14–0.65     | 1.25–1.44      |
| 4–9 years   | Mean  | –                | 42.5           | 4.88          | 18.5           |
| (n = 2)     | Range |                  | 35.0–50.0      | 3.89–5.88     | 14.2–29.8      |
| Adults      | Mean  | 70.0             | 143.2          | 13.06         | 41.9           |
| –           | Range |                  | –              | 7.17–18.94    | 32.7–51.0      |

**Table 1b.** Gastric outputs after stimulation with histamine acid phosphate (40  $\mu$ g/kg body weight) in healthy infants (9–30 months) and adults. Adapted from Rodbro et al.<sup>[64]</sup>

| Subjects    |       | Body weight (kg) | Volume (mL/hr) | Acid (mEq/hr) | Pepsin (mg/hr) |
|-------------|-------|------------------|----------------|---------------|----------------|
| 9–30 months | Mean  | 11.3             | 49             | 1.9           | 12.5           |
| (n = 18)    | Range | 8.0–13.9         | 25–105         | 0.3–3.7       | 2.2–26.0       |
| Adults      | Mean  | –                | 238            | 20.0          | 80.9           |
| (n = 12)    | Range |                  | 148–339        | 6.8–34.9      | 32.4–153.2     |

acts in the stomach of newborns,<sup>[46]</sup> since the postprandial pH in infant stomach stays above 4.5 for about two hours (Figure 2). The capacity of pepsin output after stimulation is shown in Table 1a and 1b. From birth to 3 months, pepsin secretion rises progressively, but remains well below adult levels.<sup>[55]</sup> The lower pepsin secretion coupled with higher gastric pH indicates less protein is hydrolyzed by pepsin in an infant's stomach.

Gastric emptying rate determines the length of time for interactions between hydrochloric acid, pepsin and dietary proteins. Human milk generally empties faster than infant formula, although large variations in gastric half-emptying have been reported.<sup>[12]</sup> Van Den Driessche et al.<sup>[66]</sup> reported that the mean half-emptying time was 65 (range 27–98) minutes for formula and 47 (range 16–86) minutes for breast milk. While gastric emptying is often considered slower in infants than in older children and adults, many covariates exist and this speculation may be more relevant to properties of the tested food rather than age.<sup>[67,68]</sup> Although regulations of gastric emptying are not fully understood, the type of protein is a factor that influences gastric emptying. Specifically, whey protein empties faster than casein as whey protein is soluble while casein clots in the stomach, affecting the amino acid absorption and whole body protein anabolism.<sup>[69–71]</sup> These findings have implications for infants with delayed gastric emptying and infants with low body weight, suggesting that protein composition and structure could be formulated specifically for particular infants to achieve optimal growth and metabolism.

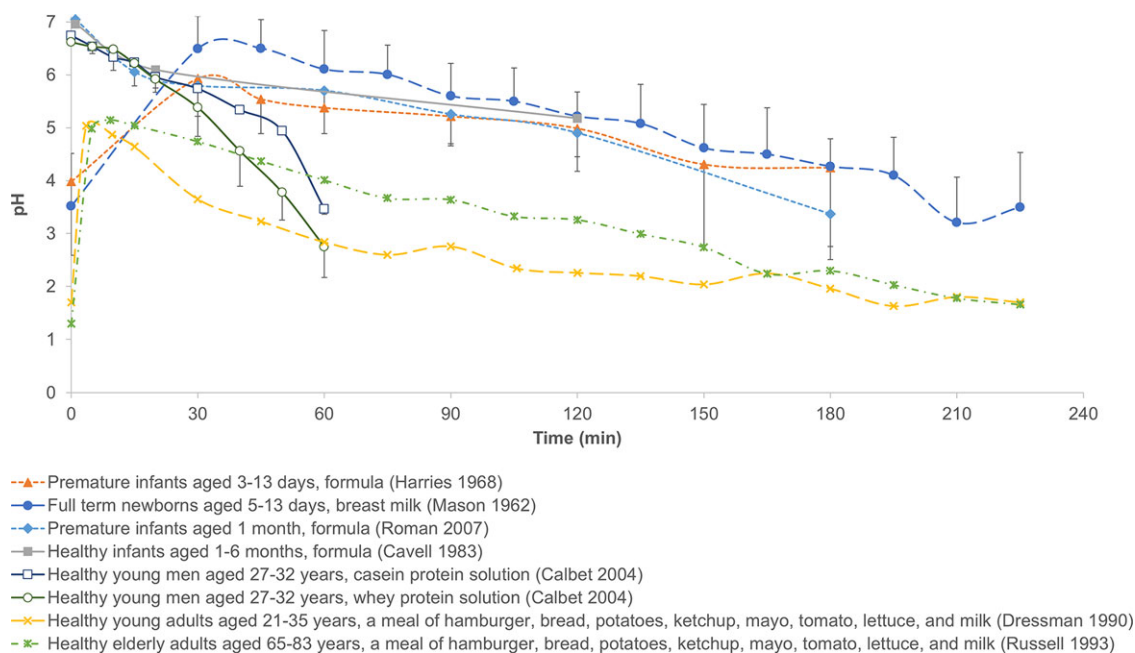


**Figure 2.** Postprandial gastric pH of healthy infants and adults.

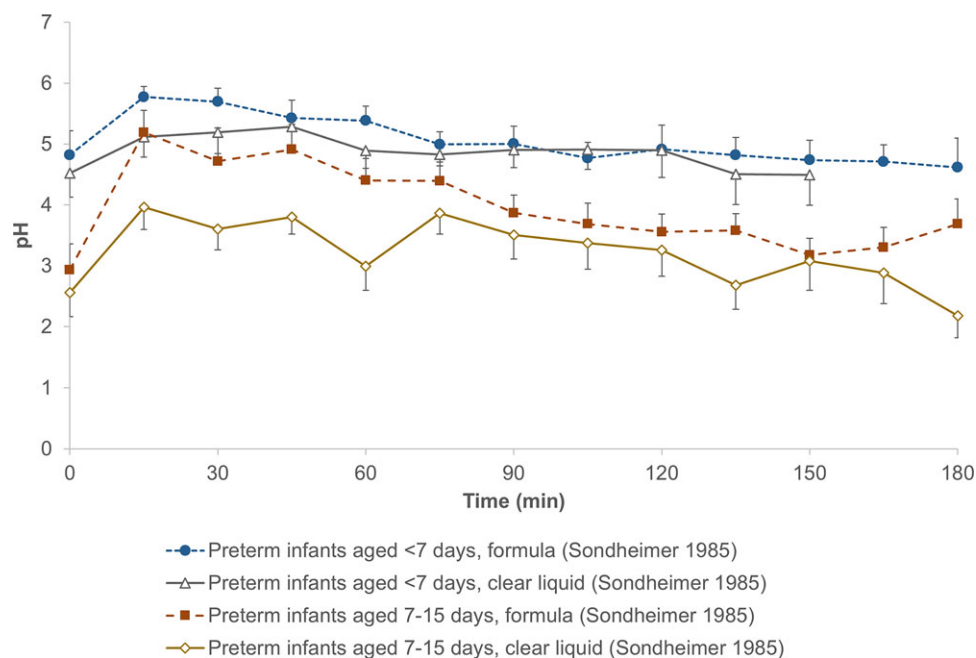


**Figure 3.** Postprandial gastric pH of preterm infants after formula and clear liquid feeding.

### 3.3. Intestinal Phase

Hydrolysis of dietary proteins begins in the stomach, while major proteolysis occurs in the small intestine.<sup>[44,72]</sup> Specificity of pancreatic proteases and their activity in infants are summarized in Table 2.<sup>[72–75]</sup> In the intestine of healthy newborns aged 3–15 days, concentrations of trypsin during fasting and after bottle-fed with breast milk were  $119.5 \pm 78.3$  and  $93.8 \pm 49.0$   $\mu\text{g/mL}$ , and concentrations of chymotrypsin during fasting and after feeding were  $100.3 \pm 66.5$  and  $86.5 \pm 45.0$   $\mu\text{g/mL}$ , respectively.<sup>[76]</sup> Lower concentrations and activities of these enzymes indicate less proteolysis occurs in infant intestine.

In contrast to different levels of enzymes in infants and adults, intestinal pH is generally similar across age groups. Bicarbonate is released from the pancreas into the duodenum to neutralize the acidic chyme from the stomach. In healthy young and elderly adults, duodenal pH is typically between 6 and 7, but it can be temporarily reduced to 5.4 after a meal.<sup>[60,62,77]</sup> Similarly, duodenal pH in normal infants and children aged from 1 month

to 12 years is 6 to 7, and the average pH of duodenal content collected within 1-hour following a 40% cream meal is between 5 to 6.<sup>[78]</sup>

Motility in the small intestine is to propel intestinal contents, mix the contents with digestive juice, and retain them long enough for digestion and absorption.<sup>[44]</sup> Differences in the amplitude of non-migrating contractions and the number of pressure waves have been observed among preterm infants, term infants and adults.<sup>[79]</sup> However, information about intestinal transit time of ingested food in infants is limited. Preterm infants (gestational age 26–33 weeks, postnatal age 6–37 days) fed with expressed human milk had gastric half-emptying time of 1.0 (range 0.5–3.0) hours and orocecal transit time of 3.1 (range 1.3–6.1) hours,<sup>[80]</sup> suggesting that small intestinal transit time is around 2 hours. The orocecal transit time has been measured in children, but results varied greatly:  $90.2 \pm 20$  minutes for children aged 1–5 years fed with 15 mL lactulose in 100 mL water,<sup>[81]</sup> and 255 (range 165–390) minutes for children aged 3–17 years fed with 250 mg lactose-<sup>[13]</sup>Cjureide in 200 mL chocolate milk.<sup>[82]</sup>

**Table 2.** Pancreatic proteases in infants.

| Enzyme            | Specificity <sup>[72]</sup>                                                                                                                                                 | Activity in infants                                                                                                                                                                                                                       |
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Trypsin           | Endopeptidase, cleaves bonds at basic amino acids - lysine and arginine                                                                                                     | In newborns (at birth and 30 days) 90% to 100% of activity in children (>2 years) after pancreozymin-secretin stimulation. <sup>[74]</sup> At birth 8% and at 1 month 25% of activity in children (9 months to 13 years). <sup>[73]</sup> |
| Chymotrypsin      | Endopeptidase, cleaves bonds at aromatic amino acids - phenylalanine, tyrosine, tryptophan                                                                                  | In newborns (at birth and 30 days) 50% to 60% of activity in children (>2 years). <sup>[74]</sup>                                                                                                                                         |
| Elastase          | Endopeptidase, cleaves bonds at small amino acids - alanine, glycine, serine                                                                                                | Low activity in infancy, develops during the first 2 years of life. <sup>[75]</sup>                                                                                                                                                       |
| Carboxypeptidases | Exopeptidase, carboxypeptidase A cleaves C-terminal residues with aromatic or aliphatic side chains, carboxypeptidase B cleaves C-terminal residues with basic side chains. | In newborns (at birth and 30 days) 10% to 25% of carboxypeptidase B activity in children (>2 years). <sup>[74]</sup>                                                                                                                      |

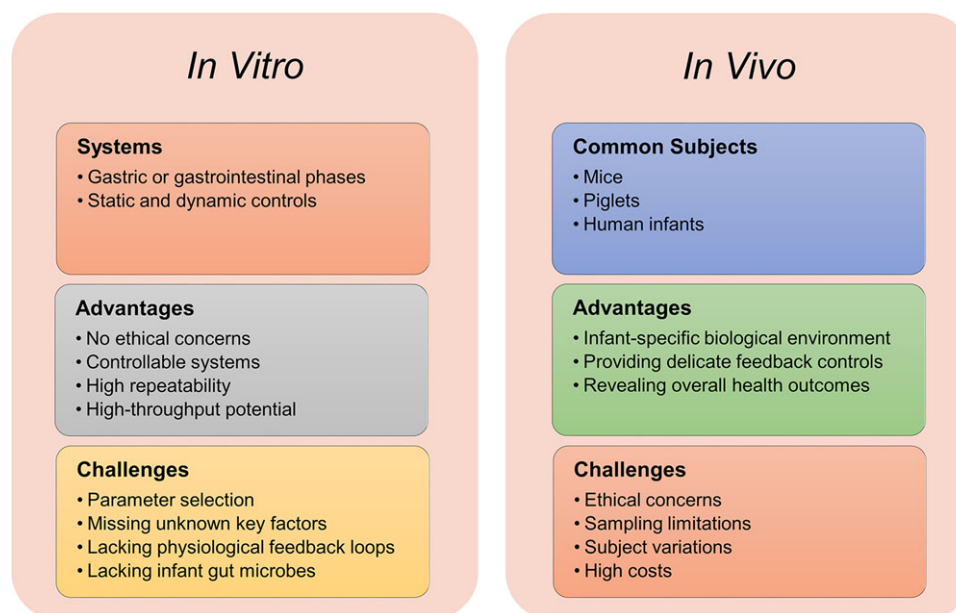


**Figure 4.** Comparison of in vitro and in vivo approaches of studies on infant protein digestion.

Assimilation of dietary proteins in the small intestine is incomplete. 5.73% of cooked egg protein and 35.10% of raw egg protein have been found to escape digestion and absorption in the small intestine in healthy adults (mean age 27 years).<sup>[83]</sup> Dietary proteins, peptides, or amino acids that escape assimilation can be fermented by gut bacteria, yielding branched chain fatty acids, amines, phenols, and indoles.<sup>[84,85]</sup> Interestingly, Piper et al.<sup>[86]</sup> measured major bacterial metabolites along the gastrointestinal tract of weaned piglets, and results suggest that considerable protein fermentation has already occurred in the stomach. However, most information is generated from animal models or fecal samples; little is known about the gut environment for protein fermentation in human infants due to limited accessibility. Some clinical studies on human infants indicate that cow's milk proteins and cow's milk formulas are associated with infantile colic, or excessive crying.<sup>[87]</sup> However, many hypotheses exist about causes of infantile colic, ranging from protein intolerance to microbiota shifts,<sup>[88,89]</sup> and yet none have been resolved since the molecular mechanism is unclear.

## 4. Approaches to Studying Infant Protein Digestion

### 4.1. In Vitro Approaches

Advantages of using in vitro models to study mechanisms of protein digestion include no ethical concerns, better control, higher repeatability, lower cost, and high-throughput potential compared to in vivo models (Figure 4). To study infant protein digestion, in vitro models should mimic conditions of the infant gastrointestinal tract that are relevant to protein digestion. Physiological conditions of the infant digestive system have been neglected in in vitro studies until the last decade, as researchers have started to establish dedicated infant in vitro gastrointestinal models. Major adjustments include higher gastric pH, reduced

enzyme concentration, and longer transit time compared to adult models.<sup>[29,40,90–96]</sup> Recent studies on this subject are summarized in Table 3.

Although parameters have been adjusted to adapt digestion models to infant conditions, the extent of adjustments vary among research groups because of limited understanding of infant physiology. Additionally, the composition and the amount of food material as well as the techniques to analyze proteolysis are diverse across studies. All these differences lead to difficulties in comparing results of protein digestion among research groups. Nevertheless, several studies offer invaluable comparisons between digestion models, revealing how varying conditions affect proteolysis.

Chatterton et al.<sup>[90]</sup> treated human milk with gastric juice from a human neonate at different pH in vitro, and meanwhile collected gastric aspirates from two infants following administration of human milk (in vivo). Analysis by electrophoresis indicated that a large amount of human milk proteins remained intact for at least 1 hour after ingestion both in vitro and in vivo, but patterns of proteolysis as shown on gels were different between these two approaches. Such discrepancy may result from the variation of samples or subjects. It may also indicate that the in vitro digestion model, using neonate gastric juice that was collected, centrifuged and frozen, missed a crucial factor that plays an important role in vivo. Dupont et al.<sup>[92]</sup> compared effects of an in vitro infant model and an adult model on the hydrolysis of purified food proteins. Their infant and adult models differed only in pH and enzyme concentrations, leading to different rates of proteolysis: ovalbumin and  $\beta$ -casein were digested slowly, while  $\beta$ -lactoglobulin was degraded more extensively in the infant model. This research group also compared kinetics of formula proteolysis in an in vitro dynamic infant gastrointestinal model to in vivo data collected from piglets. A good correlation was shown between the two models based on extent of proteolysis and residual gastric volume,<sup>[97]</sup> but whether these results correlate

**Table 3.** Recent studies on infant protein digestion using in vitro models.

| Model                                                                                       | Food                                                                                                          | Analytical techniques                                                                        | Adaptations for infants                                                                                                                                                                                                                                               | Reference  |
|---------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Static infant gastric digestion model (with porcine pepsin or human neonatal gastric juice) | Whey protein concentrate, infant formula                                                                      | SDS-PAGE, HPLC, N-terminal sequencing, western blot                                          | (1) Human neonatal gastric juice for in vitro digestion; (2) pH 6.5, 5.0, 4.0, 3.5, 3.0 and 2.0; (3) 1 hr incubation                                                                                                                                                  | [90]       |
| Static infant gastrointestinal model                                                        | Human milk with and without pasteurization (62.5 °C for 30 min)                                               | LC-MS/MS                                                                                     | (1) Gastric pH 4.0; (2) 15 min for gastric digestion; (3) 5 min for intestinal digestion.                                                                                                                                                                             | [96]       |
| Static infant gastrointestinal model                                                        | Human milk, infant formula                                                                                    | LC-MS/MS                                                                                     | (1) Gastric pH 3.5; (2) 30 min for gastric digestion; (3) 60 min for intestinal digestion.                                                                                                                                                                            | [91]       |
| Static infant gastrointestinal model                                                        | Human milk, infant formula                                                                                    | LC-MS/MS, ELISA                                                                              | (1) Gastric pH 4.0; (2) 100 U pepsin/mg protein; (3) 5 mg pancreatin and 30 mg bile salt extract/mL sample for duodenal digestion.                                                                                                                                    | [110]      |
| Static infant gastroduodenal model (with enzymes and surfactants)                           | Milk protein solutions, Maillard reaction products, food matrices (milk and yogurt) with different processing | SDS-PAGE, ELISA, immunoblotting, RP-HPLC, LC-MS/MS                                           | (1) Gastric pH 3.0 instead of 2.5; (2) 22.75 U pepsin/mg substrate (8-fold reduction compared to adult model); (3) 1 mM duodenal bile salt (4-fold reduction), 3.45 U trypsin/mg substrate (10-fold reduction), 0.04 U chymotrypsin/mg substrate (10-fold reduction). | [29,92,93] |
| Semi-dynamic infant gastric model                                                           | Model formulas with different processing                                                                      | Chemical characterization for lipids and proteins, structural characterization for emulsions | Three subsequent stages: 0–60 min at pH 6, 60–120 min at pH 5, 120–180 min at pH 4.                                                                                                                                                                                   | [40]       |
| pH static and dynamic gastric models for infants and adults                                 | Milk protein solutions, protein emulsions                                                                     | SDS-PAGE, light scattering and fluorescence microscopy                                       | (1) gastric digestion time 240 min (120 min for adults); (2) pH gradients from 6.5 to 3.5 (4.5 to 1.5 for adults) in dynamic models, and pH 2.5 in static models; (3) 210 U pepsin/mg protein (240 U for adults).                                                     | [94]       |
| Dynamic gastric model mimicking infants aged 9–12 months                                    | Human milk                                                                                                    | SDS-PAGE, LC-MS/MS, immunoblotting                                                           | (1) pH gradients from 7 to 2 over 60 min; (2) enzymatic content; (3) contraction; (4) half gastric emptying at 48 min.                                                                                                                                                | [95]       |

with data from human infants is unclear. The Lesmes group<sup>[94]</sup> tested milk proteins and their emulsions with a pH-static and pH-dynamic model for both infants and adults. They showed that proteolysis and emulsion stability differed between constant pH and dynamic pH as well as between infants and adults. Overall, varied modeling conditions alter the specificity, rate, and extent of proteolysis.

#### 4.2. In Vivo Approaches

Animal and human studies offer valuable in vivo data on food digestion. In vivo studies provide a biological environment that is hard to replicate in vitro, because the gastrointestinal tract is within a complex biological system containing delicate feedback controls. For example, secretion of digestive juice and intestinal motility in response to a meal are automatically controlled in vivo, but are difficult to reproduce through in vitro experiments. Nevertheless, disadvantages of in vivo studies include ethical concerns, sampling limitations, subject variations, and high costs. Before conducting an in vivo trial, issues such as subject selection, sample collection, and analytical techniques need to be considered. Studies for infant protein digestion on animal and human infants are summarized in Table 4.

Among common animal models, piglets are similar to human infants in terms of anatomy, histology, and digestive physiology.<sup>[98,99]</sup> Particularly, a 3-week-old piglet is comparable with a 3-month-old human infant in the activity of major proteases and fecal measures of protein digestibility; however, large differences exist between the species at birth. Moreover, the gut capacity of a 3-week-old piglet is double that of a 3-month-old infant.<sup>[98,99]</sup> Thus, appropriate physiological stage and meal portion are needed to acquire physiologically comparable results.

With animals, several methods are available for sample collection and subsequent analysis. (1) Fecal collection: this non-invasive method of sampling aims at end products coming out of the body, revealing effects on food molecules after the whole process of digestion, absorption and excretion, and is applicable to human as well,<sup>[98]</sup> offering opportunities for model validation. (2) Dynamic collection of digestive contents: digestive contents from the gastrointestinal tract can be collected through a cannula;<sup>[100]</sup> it allows information to be obtained at various time points after feeding, and reduces certain inter-individual variability, as treatments are repeatedly tested on the same animal. However, surgery and special management for animals are required, such as antibiotic treatments, which may modify their digestion process. (3) Multi-point sample collection after slaughter: collecting digested contents and tissue samples enables versatile analysis,<sup>[86,101,102]</sup> revealing the fate of food molecules along the

**Table 4.** Recent studies related to infant protein digestion using in vivo approaches.

| Subjects      | Description                                                                                 | Diet                                                                                                  | Analysis                                                                                                                                            | Reference |
|---------------|---------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Mice          | Male, weaning, n = 6 per group                                                              | Beans and rice.                                                                                       | Chemical analysis of diet and feces. Protein digestibility-corrected amino acid scores.                                                             | [111]     |
|               | 0 and 8-day-old                                                                             | Colostrum and mature milk from lactating mice.                                                        | SDS-PAGE, western blot, and amino acid sequencing of stomach content.                                                                               | [112]     |
| Piglets       | Fed from day 7 to 28, n = 28 per group                                                      | Adequate- or high-protein formulas.                                                                   | Growth measurements, hormone assays, western blot, adipocyte diameter, real-time PCR of blood and adipose tissue.                                   | [101]     |
|               | Fed from day 2 to 28, n = 18                                                                | Infant formula adapted to piglets.                                                                    | Chemical analysis, SDS-PAGE, immunoblotting, ELISA, and LC-MS/MS of content from stomach, proximal jejunum, median jejunum, and ileum.              | [102]     |
|               | Piglets weaned at 25-day-old and fed respective diets for 3 weeks, n = 6 per group          | Diets high (26%) and low (18%) in protein and with or without two different sources of carbohydrates. | Chemical analysis for major bacterial metabolites from stomach, jejunum, ileum, caecum, proximal and distal colon.                                  | [86]      |
|               | Piglets weaned at 17-day-old, and fed experimental diets from day 27 to 34, n = 5 per group | Casein diet, casein sesame expeller diet, or casein soya bean meal diet.                              | Chemical analysis of diet and ileum content for ileal digestibility calculation.                                                                    | [100]     |
|               |                                                                                             |                                                                                                       |                                                                                                                                                     |           |
| Human infants | Term, 4 to 12-day-old, n = 3                                                                | Expressed breast milk.                                                                                | LC-MS/MS analysis of gastric content collected via a nasogastric tube.                                                                              | [106,107] |
|               | Premature, 1 to 28-day-old, n = 56 in total                                                 | Human milk with an ultraconcentrated liquid human milk fortifier or a powder human milk fortifier.    | Nutritional intake analysis, growth measurements, blood analysis.                                                                                   | [113]     |
|               | Birth to 12-month-old, n = 305 in total                                                     | Low-protein formula with probiotics, standard formula, or breast milk, and complementary foods.       | Growth measurements, blood analysis for biomarkers of protein metabolism (blood urea nitrogen, insulin growth factor-1, insulinogenic amino acids). | [114]     |
|               | Premature, gestation <32 weeks, n = 60 in total                                             | Breast milk with high-protein fortifier or low-protein fortifier.                                     | Growth measurements, blood analysis for clinical chemical parameters (albumin, cystatin C, and urea).                                               | [115]     |

gastrointestinal tract and the effects of diet on blood or adipose tissues. The drawback of this method is that sample collection is only at one specific time, so studying digestion over time requires extensive resources. If resources are available, these methods of sample collection can be combined to obtain multi-aspect information that is impossible to be obtained from human infants and provide insights into the mechanism of protein digestion.

Compared to animal models, studies on human infants are constrained by ethical considerations and difficulties in subject recruitment. Most studies on infant digestion have been performed in hospitals. Hospitalization may affect infants' digestion, even though the reason of hospitalization is not related to digestive system. Anthropometric measurements can be taken on infants, such as whole body protein synthesis and breakdown, metabolic and energy balance, and sometimes blood tests.<sup>[103,104]</sup> Besides input and output measures, the process of protein digestion has been explored by analyzing contents from infant gastrointestinal tract, which were collected through nasogastric and transintestinal tubes.<sup>[59,105–107]</sup> In addition to improved methods for sample collection, advancements in analytical techniques provide more detailed information of proteolysis in human infants. In the past, total nitrogen balance was calculated from ingested food and secreted feces;<sup>[105]</sup> current analyses like liquid chromatography coupled with tandem mass spectrometry (LC-

MS/MS) have disclosed peptides that are released in infants' stomach.<sup>[106,107]</sup> Advances in in vivo studies have improved our understanding of protein digestion under physiological conditions, revealing the fate of dietary proteins and their impact on infants.

## 5. Challenges

Protein digestion of baby foods is a highly complex process. Gaining a thorough understanding of this process and revealing its implications for feeding practices are challenging. Although new technology has facilitated steady progress in the last few decades, there remain challenges limiting breakthroughs in this field:

- (1) **Comprehensive characterization of baby food properties.** Composition of baby food is often analyzed in studies, but chemical modifications of dietary proteins and physical structure of the food are less commonly considered, which are also important for protein digestion.
- (2) **Collection of detailed data from human infants.** Data collection from human infants is technically and ethically difficult. Current knowledge of infant digestion is mainly confined to the upper gastrointestinal tract, while detailed information

on the fate of dietary proteins in infants' lower digestive tract is missing, which veils the impact of diet on infant health.

- (3) **Evaluation of infant digestion models.** At present, how appropriate the measured proteolysis in an animal or in vitro model representing a human infant is undefined. Even if many in vitro parameters are set based on available infant data, critical factors are invariably missing from models. The output of protein digestion in different models has not been systematically evaluated. Without proper evaluations of research models, the translation of laboratory findings to infant feeding is restricted.

## 6. Future Directions

The emphasis of protein science is moving from amino acid provision to a dynamic nourishment and a complex bioactive signaling system. As scientists recognize the biological activities from proteins to specific peptides and their actions along the entire biogeography of the gastrointestinal tract, the role of specific proteolysis is becoming central to the success in protein nourishment – delivering beneficial functions and managing health risks such as intolerance and allergies. Improving the efficacy of protein nourishment for infants requires quantitative estimates of the performance of infant models, for example, dependence of the outcome (characteristics of proteolysis) on each independent factor (pH, enzyme concentration, bacterial contribution, etc.). The relationship of in vitro dissolution and in vivo bioavailability has been studied extensively in pharmaceuticals, which has advanced drug discoveries.<sup>[108,109]</sup> While foods are more complicated than drugs, the concept and principle of in vitro-in vivo correlation can be applied to studying food digestion, such as identifying fundamental variables which define protein digestion in infants. This information will guide the development of models that grasp a greater dimensionality of protein nourishment in time and space. New knowledge of infant protein digestion and its consequences will provide opportunities for baby food development, precise treatment, and effective diet and health management for infants.

## 7. Conclusions

Developmental events continuously take place during infancy. Food properties significantly influence protein digestion and individual health, and physiology of the infant digestive system is distinct from adult, so baby food should be developed to reflect these differences. Food and nutrition are getting to the stage where detailed and quantitative scientific knowledge will guide precise health management. Knowledge about infant protein digestion will be obtained by systematic scientific research with various approaches. The quality of research models determines the accuracy of our understanding of how dietary proteins are processed in infants. Study approaches need to be refined to consider food properties and infant specificity. Advances in understanding protein digestion of baby foods at a molecular and cellular level will empower biological discoveries and food innovations to improve infant health effectively.

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## Conflict of Interest

The authors have declared no conflicts of interest.

## Keywords

digestion model, food breakdown, infants, nutrition, proteolysis

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