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In vitro models of gut digestion across childhood: current developments, challenges and future trends

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Abstract

The human digestion is a multi-step and multi-compartment process essential for human health, at the heart of many issues raised by academics, the medical world and industrials from the food, nutrition and pharma fields. In the first years of life, major dietary changes occur and are concomitant with an evolution of the whole child digestive tract anatomy and physiology, including colonization of gut microbiota. All these phenomena are influenced by child exposure to environmental compounds, such as drugs (especially antibiotics) and food pollutants, but also childhood infections. Due to obvious ethical, regulatory and technical limitations, *in vivo* approaches in animal and human are more and more restricted to favor complementary *in vitro* approaches. This review summarizes current knowledge on the evolution of child gut physiology from birth to 3 years old regarding physicochemical, mechanical and microbial parameters. Then, all the available *in vitro* models of the child digestive tract are described, ranging from the simplest static mono-compartmental systems to the most sophisticated dynamic and multi-compartmental models, and mimicking from the oral phase to the colon compartment. Lastly, we detail the main applications of child gut models in nutritional, pharmaceutical and microbiological studies and discuss the limitations and challenges facing this field of research.

Keywords : Child; digestion; gut physiology evolution; gut microbiota; *in vitro* gut models; nutrition; pharmacy; microbiology; toxicology

Abbreviations

CoMiniGut	Copenhagen MiniGut
DGM	Dynamic Gastric Model
DIDGI	Dynamic Gastrointestinal Digester
GI	gastrointestinal
GIS1	unicameral system of *in vitro* colonic simulation version 1
GIS2	unicameral system of *in vitro* colonic simulation version 2
INRAE	French National Research Institute for Agriculture, Food and Environment
PolyfermS	Polyfermentor Intestinal Model
SCFA	Short Chain Fatty Acids
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
TIM-1	TNO gastrointestinal system 1

Introduction

The human digestion is a multi-step and regionalized process essential for health. In early life, the development of digestive functions constitutes a considerable challenge. Digestion evolves intimately with changes in feeding from birth to childhood, marked by a transition from liquid to solid diet, with a progressive qualitative and quantitative inclusion of nutrients (Buddington, 2011). The World Health Organization recommends exclusive milk intake during the first six months of life (World Health Organization, 2005). Milk consumption can continue until 2 years old but thereafter represents a minor part of the child's energy needs.

From 6 months old, pureed and mashed foods are gradually implemented up to 1 year of age, when infant diet becomes similar to the adult's one in terms of food consistency and variety (**Figure 1**). While infants are immediately able to absorb nutrients required for their growth and development, anatomical (e.g. teeth number, organ size), mechanical (e.g. chewing or gut motility) and physicochemical functions (e.g. salivary, gastric, pancreatic and biliary secretions), as well as gut microbiota composition and function, are not instantaneously mature and evolve in a child up to 3 years of age (**Figures 2 and 3**).

Due to the diversity of physiological processes occurring within the human gastrointestinal (GI) tract and their complex evolution from birth to childhood, many questions are still raised by researchers, medical doctors and industrials to better integrate the central role of digestion in the field of child related nutrition and health. What are the fate, bioaccessibility and bioavailability of nutrients, drugs or food pollutants in the child digestive tract? How do beneficial (probiotic strains) and pathogenic (food-borne pathogens) microbes survive in the child gut? How does the large variety of ingested compounds (nutrients, drugs, pollutants and microorganisms) interact with gut microbiota? Is there any link with child health or disease? The golden approach to address these questions remains human clinical trials. However, clinical investigations in newborns, infants, and toddlers are more challenging than those involving adults due to important ethical, regulatory and financial restrictions in this subset of the general population (Auby, 2012; Michael et al., 2010). In addition, such assays require invasive procedures that are restricted to end-point measurements, with difficulties to access the different segments of the GI tract (from stomach to proximal colon). Young rodent models can be used as an *in vivo* alternative to clinical studies but are also limited by major differences between animal and human diet, digestive physiology, including resident microbiota and susceptibility to infection by pathogenic microorganisms, and by dynamic differences in the first stages of life (Hugenholtz and de Vos, 2018; Walthall et al., 2005).

Alternatives to animal experiments are also widely acknowledged by the 3R (Reduction, Replacement and Refinement) European rules aiming to reduce animal experiments and favor *in vitro* alternative strategies. Among them, *in vitro* systems mimicking the child digestive environment may gain further insights into the behavior of ingested compounds throughout their GI tract. A large number of *in vitro* digestion models have been developed the last decades, ranging from mono to multi-compartmental systems and from static to dynamic ones (Cordonnier et al., 2015; Dupont et al., 2019; Guerra et al., 2012; Payne et al., 2012; Pham and Mohajeri, 2018). Nevertheless, only few of them have already been adapted to the specific conditions found in the gut of newborns (0-28 days), infants (1 month - 1 year old) or toddlers (1-3 years old).

This review describes the specificities and evolution of the healthy full-term child digestive physiology from newborn to toddlers of 3 years old, regarding anatomical, physicochemical, mechanical and microbial parameters. Then, current *in vitro* models of the child gut are described, as well as their main applications in the fields of nutrition and health. Lastly, the limitations and further challenges facing child model development are discussed.

1. Gut physiology evolution from newborns to toddlers

1.1 Digestive anatomy and functions

Changes in feeding from newborns to 3 years old toddlers require the evolution and adaptation of the whole child digestive tract anatomy allowing a more complex breakdown capacity (**Figures 1, 2 and 3**).

1.1.1 Oral cavity

At birth, the orofacial and cervical anatomy is adapted to the sucking-swallowing function, with the tongue being proportionally larger and in higher position than in older children (Delaney and Arvedson, 2008; Silva et al., 2016). During child development, the tongue gets

into a lower position as the pharynx grows, promoting the initiation of tongue's lateral movements and allowing mastication maturation (Le Révérend et al., 2014). Moreover, maxilla and mandible growth increases the oral cavity space to fracture larger pieces of food and supports increased bite and masticatory forces. The eruption of anterior primary teeth between the age of 6-8 and 18 months is an important step of oral function evolution (**Figure 1**). Around 3 years old, the complete occlusion contacts between primary molars allow the mandible to stabilize (Le Révérend et al., 2014). Finally, human salivary gland development continues across childhood, and is accompanied by several quantitative and qualitative changes in terms of saliva flow, composition and physicochemical properties (Bellavia et al., 1979; Ben-Aryeh et al., 1984).

1.1.2 Stomach

At birth, the average stomach length is 7 cm (Osemlak et al., 1982), that increases with age to finally reach 25 cm (the adult length) at around 23 years old (Begum et al., 2013). During the first days of life, the increased number of feedings induces gains in stomach compliance, followed by an increase in stomach relaxation capacity allowing a greater content (Zangen et al., 2001). At birth, parietal and mucous neck cells from gastric mucosa are well-defined, while there are few chief cells when compared to the adult stomach (Deren, 1971). In full term newborns, the number of glands and crypts remains constant until 2 and 6 months old, respectively and rises right after to reach adult values at around 1.5 years and 5 years, respectively.

1.1.3 Small and large intestine

The length of the small intestine is increasing from birth (275 cm) to reach 400 cm at 4 years old and then 350-500 cm in adults (Gondolesi et al., 2012; Weaver et al., 1991). All intestinal cell types are fully developed at birth (Moxey and Trier, 1978). There is no available data on how absorption surface area and villus length evolves in the first years of age in child. After

birth, 6 months old children exhibit a colon of 568 mm ± 27 mm in average, reaching 1224 mm ± 57 mm in average at 4 years old. The elongation process stops around 5 years old with a length of around 1300 mm (ranging from 1100 to 2108 mm), similarly to adults (Wozniak et al., 2019). The mean relative length of the different colonic segments (ascending, transverse and distal) shortly varies compared to the total colonic length after birth (Mirjalili et al., 2017).

1.2 Mechanical digestion and transit time

1.2.1 Food mastication

To date, food fragmentation has been poorly studied in children (Barrera et al., 2011; Gavião et al., 2007), and the bolus state at the time of swallowing before 3 years old is unknown..

Lips pressure increases over age (**Figure 2**), particularly between 5 months and 3 years old (Chigira et al., 1994). Food is usually mashed by the tongue in upward/downward movements at 4-6 months old (Ayano et al., 2000). At 10 months, children start to move solid food from one side of the mouth to the other using tongue lateral movement (Stolovitz and Gisel, 1991). Following introduction of hard consistencies food, horizontal jaw motion during chewing becomes more observable at 2 years old. Rotational movements typical of a mature chewing can be observed at 2.5 years old (Arvedson and Lefton-Greif, 1996; Ayano et al., 2000; Takada et al., 1994; Wilson et al., 2012). Muscle activity during mastication of different food matrices in infant increases between 8 and 22 months, to reach a level similar to that observed in adults (Green et al., 1997; Steeve and Price, 2010).

1.2.2 Gastric digestion

Mixing waves begin in the stomach following food entry and fasting antral motor patterns do not differ between adults and infants (Broussard, 1995). Gastric emptying time of newborns seems longer compared to adults even if there is no absolute consensus. In newborns, it extends from 10 to 100 min following milk consumption with a mean half-time emptying of

87 min *versus* 65 min in healthy adults (Signer, 1975). Besides, formula-fed infants displayed a longer gastric emptying time compared to breast-fed infants (78 *versus* 48 min), due to a greater amount of casein in formula (Cavell, 1979). Yet, a meta-analysis showed contrasting data with no observed difference in gastric emptying rate and time between different postnatal ages (i.e. birth–1 month, birth–23 months, 2–5 years, 6–12 years, 13–18 years), even for a variety of food types (Bonner et al., 2015).

1.2.3 Intestinal digestion

All the intestinal muscles and neural structures appear before birth, while neuroendocrine transmission and integration are not completely achieved at that time and continue to mature during the first year of life (Berseth, 2006). Endoscopic analysis performed in a cohort of infants ranging from 10 months to 9 years displayed a median small bowel transit time of 401 min (ranging from 264 to 734 min), while in adults with mean age of 59 years old, a reduced transit time of 283 min (from 91 to 416 min) was reported (Oikawa-Kawamoto et al., 2013; Velayos Jiménez et al., 2005). Between birth and 2 years old, colonic transit time and stool frequency decrease, within a mean of 4 stools per day at 1 week old, 2.2 after 1 month and 1.7 stools after 1 year (Lemoh and Brooke, 1979). After 2 years old, the mean colonic transit time is not significantly different from adults (mean 31.78 hours from various studies) as measured with radio-opaque plastic markers according to a meta-analysis (Southwell et al., 2009). In addition, stool frequency differs with diet, breast-fed infant producing daily less dry feces weight than formula-fed infant (Sievers et al., 1993).

1.3 Digestive secretions and pH

Digestive secretions also undergo deep maturation closely related to feeding evolution during childhood (**Figure 3**).

1.3.1 Saliva

During infancy, saliva undergoes manifold changes closely dependent on tooth eruption and food transition from milk to progressive solid food (**Figure 2**). Sodium, potassium and chloride quantities decrease from birth and during the first year of life (Ben-Aryeh et al., 1984). Among a marked steadiness in the overall pattern of salivary proteins during child growth, mucin secretion displays variations in terms of types of molecule with age, with a shift in predominance from MUC7 to MUC5B (Ruhl et al., 2005). Very low levels of α-amylase activity were reported at birth (Ben-Aryeh et al., 1984; Rossiter et al., 1974), from 6 to 9 times lower compared to adults (Bellavia et al., 1979). However, a rapid and strong increase in salivary α-amylase activity was reported, reaching adult levels at 6 months (Ben-Aryeh et al., 1990, 1984; Rossiter et al., 1974). Milk and high-fat diet of newborns require an adequate lipolytic activity. Due to a low pancreatic lipase activity in newborns and infants, salivary lingual lipase has probably a specific role in digestion of fat from milk (Fredrikzon et al., 1982; Hamosh, 1979; Hamosh et al., 1981). With growth, a progressive shift from lingual lipase activity toward an increasing role of pancreatic lipase has been reported (Lebenthal et al., 1983). Saliva in babies under 1 month is highly viscous with a very low flow rate that increases with age to reach 0.37 mL/min in adults (Sinor et al., 2009). Saliva is also slightly more acidic at birth (pH 6) than in adults (pH 6.33) (Ben-Aryeh et al., 1984).

1.3.2 Gastric secretions

Lipid digestion in the stomach is a critical step for newborn to offset the lack of pancreatic lipases and bile acid production (Hamosh et al., 1981). At birth, gastric lipase activity already reaches adult level (Lindquist and Hernell, 2010) and fat gastric digestion leads in newborn, similarly to adults, to the hydrolysis of 10 to 30% of total esterified fatty acids (Mu and Høy, 2004). On the contrary, protein digestion is impaired in newborns due to the combined effect of low pepsin secretion (at 3 months pepsin concentration represents half of the adult one) and

activity (only 13% of adult level at birth) and higher post-prandial gastric pH compared to adults (Agunod et al., 1969; Bourlieu et al., 2014). Despite the absence of studies related to mucus secretion and characteristics in child, newborns seem more vulnerable to gastric acidity, which could be linked to a lower gastric mucosa thickness during the 3 first weeks of life (Bourlieu et al., 2014). To favor protein and lipid digestion by gastric pepsin and lipase, the stomach secretes hydrochloric acid to maintain a low pH. Before meal ingestion, between 5- and 13-days full-term infants have a mean pH of 3.5 (ranging from 2.0 to 6.1). Gastric pH increases in average to reach 6.4 after breast milk ingestion and remains elevated up to 60 min following meal ingestion, to decrease progressively to reach the pre-feed state pH (Mason, 1962). Therefore, like in adults, gastric pH increases after food ingestion in child, but remains higher for a longer period (Hamosh, 1996). Accordingly, this high pH range could be explained by a low acid production by infant parietal cells, reaching adult value at 6 months, combined with the high buffering capacity of milk (Dallas et al., 2012).

1.3.3 Pancreatic, liver and intestinal secretions

Pancreas and liver, together with the small intestine, release digestive secretions in order to degrade remaining proteins, lipids and carbohydrates into small polymers and monomers ready for absorption (**Figure 3**).

Pancreatic amylase is not detectable before the first month of life and reaches adult levels by 8 months old (Otsuki et al., 1977). On the contrary, intestinal saccharase-isomaltaseis already present at birth (Triadou and Zweibaum, 1985). In response to milk feeding, intestinal lactasereaches its maximal activity at birth and thereafter decreases to 5-10% of its initial activity during childhood and adolescence (Drozdowski et al., 2010; Montgomery et al., 1999). The expression of pancreatic proteolytic enzymes reaches adult levels few weeks after birth. Duodenal enterokinase, the enzyme activating trypsinogen into trypsin, which in turn activates pancreatic proteases, shows at birth 20% of the activity found in infants and toddlers

(1-4 years old) (Antonowicz and Lebenthal, 1977). The brush borders peptidases, aminopeptidase and dipeptidyl-peptidase IV, already reach adult activity levels at term (Auricchio et al., 1981; Lacroix et al., 1984). Pancreatic lipase displays an activity that remains low at birth to increase from 10 weeks of life and reach the adult one by 3 years of age (Cleghorn et al., 1988).

At birth, bile salts concentrations in duodenal juice remain very low, representing in average 1.8 mmol/L under 2 days of age. From 10 days to 7 months, it rises up to reach 9.8 mmol/L. Bile composition switches from mainly taurine-conjugated bile salts at 7 days to glycine-conjugated bile salts after 10 days (Challacombe et al., 1975). This low bile acids pool could explain fat malabsorption in young infants leading to a greater fat delivery into the colon (Murphy and Signer, 1974; Watkins et al., 1974). Primary bile salts are released into the duodenum, and following their action on lipid emulsification, they reach the ileum to be re-absorbed. During the first week of life, cholic acid is the major primary bile acid with a 2 to 5:1 ratio with chenodesoxycholic acid, the other main primary bile acid. However, during the first month, this proportion falls to 1-2:1, which is also the ratio found in adults (Murphy and Signer, 1974). Formation of secondary bile salts requires enzymatic modifications (deconjugation and dehydroxylation) of primary bile salts by gut microbiota. Of note, no secondary bile salt is extracted from the feces of newborns (Sharp et al., 1971) and the ratio between primary and secondary bile acids changes in the colon along with gut microbiota development (Neal-Kluever et al., 2019).

1.3.4 Colonic secretions

There is no available data on the evolution of mucus composition in the colon in the early age. The intestinal mucin genes are expressed early before birth but a fully developed and protective mucus barrier is formed a couple of days after birth (Pelaseyed et al., 2014). Regarding infant colonic pH, no data is available and only fecal pH value was reported. Fecal

pH varies a lot with age after birth and between individuals according to the evolution of both diet and gut microbiota composition and activity (Bullen et al., 1977; Henrick et al., 2018). It gradually increases with age, with an average fecal pH at 3 days around 5.3 ± 0.6, reaching 5.7 ± 0.7 for 6-month infant (George et al., 1996), then 5.8 ± 1.1 between 12 to 18 months (Edwards et al., 1994), to be ultimately similar to that of healthy adults with a value around 6.6 ± 0.3 (Osuka et al., 2012).

1.4 Intestinal absorption of nutrients and water

In the small intestine, glucose and galactose released from lactose digestion are absorbed by enterocyte transporters that are already expressed in newborns (**Figure 3**). On the contrary, fructose-specific transporter is only expressed from 1 year after birth. All amino-acid transporters are functional at birth (Drozdowski et al., 2010; Malo, 1991). Similarly, the predominant apolipoprotein 48, forming chylomicrons with phospholipids and triglycerides and involved in lipid absorption, is early expressed during gestation (Glickman et al., 1986). At birth, all vitamin and mineral absorption mechanisms are mature (Abrams et al., 1997). Nevertheless, vitamin K and D storages are very low at birth, as well as sunlight exposure and gut microbiota activity, making necessary vitamin supplementation (Salle et al., 2005). Moreover, due to the weak bile salt pool during the first 2 months, absorption of fatty acids and liposoluble vitamins can be impaired (Hamosh et al., 1981). One of the main functions of the colon is to absorb water, electrolytes and short chain fatty acids (SCFA) resulting from microbial fermentation, but also to maintain normal fluid homeostasis, in particular in newborns still displaying immature small intestinal absorption functions (Pácha, 2000). In particular, neonates have a greater nutrient absorptive capacity in the colon than adults, due to the presence of apical brush border hydrolases in the colonic mucosa during the first week of life (Lacroix et al., 1984; Pácha, 2000).

2. Oral and intestinal microbiota evolution: from birth to 3 years

2.1 Colonization and composition of infant microbiota

Development of resident communities and their metabolic outcomes are crucial not only for early life maturation, but also for long-term health in children, and to a later extent in adults. Gut microbiota is also mandatory for immunologic programming of infants (Dzidic et al., 2018; Wiertsema et al., 2021) and exerts a key barrier effect against pathogens (Vazquez-Gutierrez et al., 2016).

Microorganisms rapidly colonize infants over the first months or years of life. Currently, no consensus exists concerning the time for microbiota maturity but most studies converged on the time period of 1000 days, i.e. three first years of life (Derrien et al., 2019; Odamaki et al., 2016). Of note, the only available data on resident microbiota in the first years of life are related to oral and fecal microorganisms and high individual variations in microbiota composition and functions have been reported (Könönen, 2000; Pham et al., 2016). Also, the bacterial fraction has been extensively investigated, while little is currently known on the other microbial communities, i.e. fungi, Archaea and viruses (Korpela and de Vos, 2018; Zaura et al., 2009). Gut microbiota of at term, vaginally born and exclusively breast-fed infant is recognized as healthy infant microbiota (Arboleya et al., 2015).

The colonization process occurring in infants is characterized by a continuous increase in load and diversity of bacteria during the first year of life, and even later on, up to the weaning time (Cilieborg et al., 2012; Pham et al., 2017, 2016; Sulyanto et al., 2019). A wide range of species have been identified in the oral microbiota of child below 3 years old, most of these species being more frequently present on the tongue in babies below 18 months old (Tanner et al., 2002). In early childhood, infants share 85% of their oral microbiota with their mother, pointing out the importance of maternal oral microbiota (Mason et al., 2018). Microbiota personalization begins with diet changes and teeth eruption that creates new

microenvironments (Kennedy et al., 2019; Könönen, 2000; Tanner et al., 2002). From birth to 6 months, Firmicutes and especially *Streptococcaceae* dominate the oral community, as well as the genera *Actinomyces*, *Gemella*, and *Veillonella* (Könönen, 2000; Lif Holgerson et al., 2011; Pearce et al., 1995; Sulyanto et al., 2019) (**Figure 3**). During the following years until 3 years old, abundances of Firmicutes (*Streptococcaceae* and *Veillonellaceae* families) and Actinobacteria decrease, while Proteobacteria, Fusobacteria and Bacteroidetes increase (Crielaard et al., 2011). The only available study on other non-bacterial populations reports a poor fungal colonization during the first months of life with *Candida parapsilosis*, *Saccharomyces cerevisiae* and *Candida albicans* (Ward et al., 2018). Regarding intestinal microbiota, aerobic and facultative anaerobes are the first colonizers in the neonatal gut due to the presence of oxygen, which limits the colonization of strict anaerobes (**Figure 3**). Fecal microbiota mainly consists of *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Lactobacillus* and *Enterobacteriaceae* (mainly *Escherichia coli* and related species). Those facultative anaerobe bacteria contribute to the establishment of a reduced environment that is mandatory for strict anaerobes colonization. This important shift seems to occur at 4 months old (Bäckhed et al., 2015) and colonization process of anaerobic microbes belonging to the predominant phyla Bacteroidetes, Firmicutes and Actinobacteria occurs then very quickly. The presence of *Bacteroides* and/or *Bifidobacterium* species that outnumber facultative anaerobes and reach adult-like population densities within the first week of life is even reported from fecal sample analysis (Jost et al., 2012). Both *Bacteroides* and *Bifidobacterium* species are well-known for using most of the substrates available in the neonatal gut from infant milk-based diet (proteins, lactose but also human milk oligosaccharides) and host-produced components such as mucin glycoproteins (Jost et al., 2015), which could explain this rapid establishment. Another stage of intestinal colonization occurs after several weeks or months, together with an increase in microbial diversity. Some species belonging to

Firmicutes (mainly *Lachnospiraceae* and *Ruminococceae*) significantly increase over the first year of life in stool samples. Increase in microbial diversity is further associated to diet changes after weaning (Yassour et al., 2016) and for several years. Available reports on fecal samples suggest an early colonization of bacteriophages, but a delayed colonization of fungi, and especially *Archaea* even beyond the weaning time (Korpela and de Vos, 2018; Vanderhaeghen et al., 2015).

2.2 Impact of external factors on oral and intestinal microbiota

Gestational age can strongly affect gut microbiota development. A decrease in gestational age leads to a delayed colonization of strict anaerobes and a lower diversity, together with an increase in potential pathogenic and facultative anaerobic bacteria (Henderickx et al., 2019). Direct comparison of gut microbiota composition of preterm and term infants showed that *Enterobacter*, *Enterococcus*, *Escherichia* and *Klebsiella* populations, containing well-known opportunistic pathogens, were predominantly present in preterm infants (Arboleya et al., 2012; Henderickx et al., 2019). Environmental and host conditions can partially explain these microbial differences in colonization, together with specific feeding regimen and the frequent use of antibiotics associated with prematurity (Henderickx et al., 2019).

Consistent evidence also indicates that the delivery mode has a significant impact on infant oral and gut microbiota colonization profiles. A higher number of bacterial taxa was reported in the oral microbiota of vaginally born compared to caesarian section born infants, with two distinct colonization patterns, even if these differences attenuate after 1 month (Hurley et al., 2019). Oral microbiome is dominated by *Slackia exigua* (periodontitis-associated species), *Streptococci mutans* and *Lactobacillus Cluster I* in caesarian born infant, while *Haemophilus parainfluenzae* is predominant in vaginally born individuals (Li et al., 2005; Lif Holgerson et al., 2011). Regarding intestinal microbiota, the primary gut colonizers of babies born vaginally show a high microbial similarity with their mother's vaginal microbiota dominated

by *Lactobacillus* and *Prevotella* species. By contrast, this natural exposure is disrupted in caesarean born babies and the primary colonizer species are mostly *Staphylococcus* and *Propionibacterium*. This suggests an initial colonization with microbes found in the hospital environment and later from skin contact with mother as origin (Dominguez-Bello et al., 2010).

The feeding mode also strongly affects infant oral and gut microbiota colonization. The comparison between breast-fed and formula-fed babies was largely investigated over the past decades. At 3 months, breast-fed infants harbor a high amount of *Streptococcus* in their oral microbiota while *Actinomyce*s and *Prevotella* prevail in formula-fed infants. Later, the implantation of solid food in the infant diet leads to a greater oral bacterial richness compared to only breast-fed or mixed (breast and formula fed) diet, while no difference was reported for the fungal population (Oba et al., 2020). At last, feeding mode in the first months seems to have long-term effects as some differences still persist in bacterial diversity and profiles in teenagers (Eshriqui et al., 2020). Feeding mode also impacts intestinal microbiota as *Lactobacillus* and *Bifidobacterium* species are more prevalent in breast-fed infants than in formula-fed infants, associated with a decrease in their fecal pH (5.9 in breast-fed compared 6.8 in formula-fed infants) 2 weeks after birth (Simhon et al., 1982). By contrast, a more diverse gut microbiota was observed in formula-fed infants with higher level of *Bacteroides*, *Clostridium* and *Enterobacteriaceae* compared to breast-fed infants (Zhuang et al., 2019).

Besides, drugs and especially antibiotics can also profoundly influence infant gut microbiome. Impact of antibiotics on infant gut and oral microbiota obviously depends on the spectrum, dose, duration and route of treatment (Wang et al., 2016). Maternal intrapartum antibiotic treatments lead to a shift in infant oral and gut microbiota, with a loss of beneficial commensal populations (e.g. *Streptococcaceae* and *Bifidobacteriaceae*) for the benefit of *Proteobacteria* (Gomez-Arango et al., 2017; Zimmermann and Curtis, 2020). Similarly, oral

use of antibiotics in infants resulted in decreased numbers of gut anaerobic communities, particularly *Bifidobacterium* and *Bacteroides* species (Penders et al., 2006) and/or increased numbers in *Proteobacteria* such as *Enterobacteriaceae* (Arboleya et al., 2015). Intestinal communities in antibiotic-exposed infants are also less mature compared to those of unexposed ones (Bokulich et al., 2016). Lastly, excessive hygiene conditions during early life reduce the continuous microbial exposure which is however essential for the development of a rich gut microbiota in infants (Neu and Rushing, 2011; Schmidt et al., 2011).

2.3 Gut microbiota activity

The infant gut microbiome metabolizes the complex substrates of milk-based diet, such as proteins, lipids and carbohydrates (especially human milk oligosaccharides) into key metabolites such as SCFAs. The only available data on SCFA production have been obtained from child fecal samples. Whatever the feeding status, total SCFA concentrations found in infant feces between 3 to 5 months were 142 µmol/g (101.6–203.8) in average with a majority of acetate (80%) and less propionate (10%), butyrate (5%) and valerate (2%) (Bridgman et al., 2017). Another more recent study measuring only the three main SCFAs reports a median total concentration of 84.6 µmol/g of feces in 1-year old children, allocated in 72% acetate, 16% propionate and 12% butyrate (Roduit et al., 2019). These authors underline that fecal SCFA concentrations deeply depend of the infant diet. In adults, total SCFA concentration represents an average ranging from 64 to 77 µmol/g of feces depending on the study (Baxter et al., 2019; Høverstad et al., 1984), with percentages of around 60%, 20% and 20% for acetate, butyrate and propionate, respectively. Relative percentages of fecal acetate/propionate/butyrate in child are therefore far from the ideal adult ration of 3/1/1 (den Besten et al., 2013). Breast-fed infants have a lower concentration of fecal SCFAs (99.4 *versus* 177.8 µmol/g) and a higher relative proportion of acetate (86.7 *versus* 77.3 %) and lactate (7.2 *versus* 2.3 µmol/g) compared to formula-fed infants at 1 month. This is probably

due to the more efficient capacity of breast-fed infant microbiota to metabolize human milk substrate (Bridgman et al., 2017) and the greater concentration of oligosaccharides in breastmilk compared to formula (Bode, 2015).

3. Current *in vitro* models of the child gut

Even though *in vitro* gut models cannot obviously integrate nervous or endocrinal systems, they can reproduce all or part of the following physicochemical and microbial parameters of the child's digestive tract when simulating a liquid or solid food intake: temperature, pH, transit time, mechanical constraints, supply of digestive enzymes and bile salts, passive absorption of nutrients and water, condition of oxygenation (especially anaerobiosis in colonic compartments) and complex, metabolically active and compartmentalized resident microbiota from human origin (**Figure 4**). The main child *in vitro* digestion models currently available (from birth to 3 years old) are described in the next sections and summarized in **Table 1**, their applications being further explained in section 4.

3.1 Models of the oral phase

Despite its importance in child digestion physiology, very few *in vitro* models are reproducing the oral cavity. These models have been mainly designed for pharmacological studies. Gerrard's team from University of Cambridge in UK developed an *in vitro* model mimicking typical suction rates and feed volume in breastfeeding for infant under 6 months of age (Gerrard et al., 2013). A silicone human nipple mimic (HNM) was constructed with ducts to simulate dynamically the breast milk flow, as induced by infant suction. The model has been adapted to the testing of tablets containing a highly soluble dispersive formulation. In practice, heated human milk is pumped from a reservoir into the silicone human nipple mimic model, where the tablet releases the tested component into milk, then removed with a vacuum

pump. The suction pulse rate, milk flow rate, milk composition, as well as tablet formulation, can be adapted according to various scientific and/or application-driven questions. More recently, another *in vitro* model, the Tongue Mimic System (TMS), reproducing 1 to 3 months old infant peristaltic tongue motion and pressures during breastfeeding, was also developed by University of Cambridge (Scheuerle et al., 2017). In this model, heated fluids (water or milk) are pumped from a reservoir to undergo tongue movements simulated by a peristaltic pump. Tongue motions can be adjusted by modifying the shaft's rotation rate through variations of the pump speed. A movable metal plate also reproduces the hard palate of infant mouth. As described for the HNM system, this model can be loaded with a tablet.

3.2 Gastric and small intestinal models

Some of the gastric and small intestinal models firstly developed for adult conditions have been adapted to child physiology. In the following section, these models of increasing complexity are described from static mono-compartmental to dynamic multi-compartmental devices.

3.2.1 Static mono-compartmental models

A large number of simple static models of the child stomach and small intestine have been developed yet. In these models, the successive steps of digestion are reproduced in a single vessel by changing pH and enzymes/bile conditions between gastric and intestinal phases, mostly without taking into account the time effect on these parameters.

The first static child model was developed in 2010 by Dupont and colleagues from the National Research Institute for Agriculture, Food and Environment (INRAE) in France (Dupont et al., 2010). The model was adapted to 2 weeks - 3 months year old infants. During the gastric phase, pH was adjusted at 3 instead of 2.5 for adult protocol and pepsin concentration was decreased 8-fold. In the intestinal phase, bile salts amount was reduced 4-fold and phosphatidylcholin, trypsin and chymotrypsin concentrations were decreased by 10-

fold compared to adult conditions. Then, the adult international consensus protocol of *in vitro* static digestion (Minekus et al., 2014) was adapted several times to specific child conditions (Cattaneo et al., 2017 ; Ménard et al., 2018). In particular, Dupont's team set up a new static *in vitro* model for full-term newborns (28 days of life) (Ménard et al., 2018). In this model, both gastric and intestinal phases lasted 60 min, as for adult conditions. However, pH values were adapted, with a gastric pH increased to 5.3 (*versus* 3 in adult) while intestinal pH was reduced to 6.6 (*versus* 7 in adult). Gastric pepsin was largely reduced (268 U/mL *versus* 2000 U/mL) while gastric lipase remained equivalent to adult concentration and only slightly adapted to infant body weight (19 U/mL *versus* 21 U/mL). Intestinal juices reproduced by porcine pancreatin and bovine bile were largely reduced, with a 22-fold and 3-fold reduction compared to adult levels, respectively.

Very recently, another gut model was implemented by Hunan Agricultural University in China, for infants ranging from birth to 6 months old (Luo et al., 2019), based on the publication from Bourlieu and colleagues (Bourlieu et al., 2014). Of note, this model integrates kinetics of gastric pH (with four steps: pH 6.5 from 0–30 min, 6.0 from 30–60 min, 5.5 from 60–90 min and 5.0 from 90–120 min), allowing to mimic the slower pH decrease in infant compared to adult. Intestinal digestion was set up at pH 7 as in other models. The composition of simulated gastric and intestinal fluids was also adapted to infant conditions. The gastric phase was mimicked with 0.4 mg/mL pepsin and 0.87 mg/mL lipase, both added at the beginning of each pH steps. During the intestinal phase, 5 mg/mL of porcine bile extract and 1.6 mg/mL of porcine pancreatin and porcine pancreatic lipase were added.

Kamstrup's team, from University of Copenhagen in Denmark, also provided advices to design an improved gut model for neonates under 2 months old (Kamstrup et al., 2017). This model aims to integrate various parameters, such as volume, pH, osmolality, gastric pepsin and lipase, intestinal lipase and bile salts, and distinguishes fasted state from fed state. During

fasted state, gastric phase pH was set at 2.9, while a pH kinetic was considered during the fed state increasing to 6.4, 30 min after feeding and decreasing to 3.9, after 50-min digestion. For the intestinal phase (2h), the pH was maintained at 6.5 during fed state compared to 7.0 for fasted state. For the fasted state, an osmolality of 298 and 308 mosmol/kg of intestinal content was recommended for the gastric and intestinal phases respectively, compared to 291 and 296 mosmol/kg for the fed state. In gastric fluids, lipase activity was set up at 100 tributyrin units (TBU)/mL while pepsin activity was 63 U/mL/kg of body weight. Lastly, in intestinal fluids, lipase activity was set at 50 TBU/mL and bile salts concentration at 1 mM and 4.7 mM for fed and fasted states, respectively.

From Kamstrup's work, Klitgaard and collaborators developed the immediate transfer digestion model (ITDM) reproducing the stomach and small intestine of 0 to 2 months newborns (Klitgaard et al., 2017). They used the same parameters, as previously described (Kamstrup et al., 2017), except for the gastric pH (adjusted at 6.4), and the use of pancreatic extract instead of intestinal lipase.

Finally, a dissolution model was developed and adapted from the existing dissolution paddle apparatus, the United States Pharmacopeia (USP) apparatus III of adults to simulate pH conditions and fluid volumes of the stomach, small intestine and proximal colon of infant (Karkossa et al., 2017). Intriguingly, the simulated age was not mentioned in this study. Two heated-controlled vessels were filled with the infant adapted dissolution media, . The gastric phase was reproduced in one vessel during 30 min. At the end of this phase, all the gastric vessel content was transferred into a second vessel for the intestinal and proximal colonic phase by adding the specific intestinal media (lasting 4h for the small intestine and 8h for the proximal colon). Gastric pH was either set at 1.8 or 4 in two different experiments to test the gastric pH range reported *in vivo*, while the small intestinal and proximal colonic pH were maintained both at 6.8. For the gastric medium, a simulated gastric fluid without pepsin

(SGFsp), at pH 1.8 was considered. The intestinal medium is reproduced by a pH 6.8 bicarbonate based simulated intestinal fluid (Carbonate-SIF, CarbSIF) containing 120 mM sodium chloride and 5 mM potassium.

To summarize, an increase in gastric pH use of lower digestive volumes and a decrease in pepsin and intestinal lipase activities and bile salts concentrations, are the marked parameters changed in static infant models compared to adult conditions. These simple models can be used for large screening of active compounds due to their easy manipulation and low cost, but digestive conditions that are reproduced are far from mimicking the *in vivo* complexity of the infant/child digestive tract.

3.2.2 Dynamic mono- or multi-compartmental models

Compared to static models, dynamic systems integrate the evolution of digestive parameters over time and/or chyme transit between successive compartments.

3.2.2.1 Mono-compartmental models

Israel Institute of Technology developed a gastric model for infant under 2 years of age (Shani Levi et al., 2013). A unique vessel is water-jacketed, temperature-controlled, continuously stirred and filled with a gastric medium containing sodium chloride, hydrochloric acid and pepsin. Time of gastric digestion was increased in infant compared to adult (4 *versus* 2h). pH kinetics during the gastric digestion was monitored and adapted to infant digestive conditions: 6.5 (0-30 min), 6.5 (30-150 min), 4.5 (150-240 min) and 3.5 at 240 min. Lastly, pepsin concentration was reduced compared to adult level (210 *versus* 240 U/mg protein).

3.2.2.2 Bi-compartmental models

The first bi-compartmental model set-up for infant conditions (birth to 6 months neonate) was adapted from that developed by INRAE in France for adult and named DIDGI® for Dynamic Gastrointestinal Digester (Ménard et al., 2015, 2014). Two consecutive temperature-controlled compartments are reproducing the stomach and small intestine. Interestingly, a

Teflon membrane (2-mm holes) reproduces the pylorus sieving effect. Several sensors allow the control of temperature, pH and redox potential and variable-speed pumps ensure meal flow monitoring, compartment emptying, and supply of hydrochloric acid and sodium carbonate, bile and digestive enzymes. The volume and flow rates of secretions were adapted from another multi-compartmental infant *in vitro* model -described later in this review- (Blanquet et al., 2004) or from piglet *in vivo* data, with 1,250 U/mL pepsin (0.25 ml/min) and 60 U/mL lipase (0.25 mL/min) in the stomach compartment, and 1% porcine bile (0.5 mL/min) and 10% porcine pancreatin (0.25 mL/min) in the small intestinal compartment. In the gastric compartment, half time gastric emptying was set at 70 min based on human *in vivo* studies (Bourlieu et al., 2014), while the pH followed an acidification profile based on piglet data. The intestinal half time emptying was set at 200 min according to human data (Bourlieu et al., 2014) and pH was maintained at 6.5. It should be emphasized that the validation of the infant model based on protein digestibility was performed based on *in vivo* piglet studies and not humans' ones.

Klitgaard and collaborators also improved their immediate transfer digestion model (see section 3.2.1) into a dynamic system simulating infant stomach and small intestine (0 to 2 months) called the Continuous Transfer Digestion Model (CTDM) (Klitgaard et al., 2017). The same parameters as previously described were implemented but a continuous transfer of GI fluids was set up between two distinct vessels reproducing the gastric and small intestinal compartments. Gastric and intestinal digestions started together and the transfer rate between the two vessels was adjusted to mimic gastric emptying of neonates. The GI transfer ends after the complete emptying of the gastric vessel (around 150 min) and intestinal digestion is stopped later at 180 min. Interestingly, the CTDM was adapted to incorporate the gastric pH drop following meal ingestion based on an old report from Mason (Mason, 1962): gastric pH

is maintained at 6.4 during the first 30 min and then undergoes a gradual drop to 5.1 (30-120 min).

3.2.2.3 Multi-compartmental models

The digestive complexity was further improved in the Model of an Infant Digestive Apparatus (MIDA) developed by University of Naples in Italy (Passannanti et al., 2017). The four compartments reproduce the esophagus, stomach, pyloric sphincter and small intestine of 6-month infants. Each of them, except for the pyloric sphincter, is represented by two concentric tubes, the inner containing the digestive medium while the outer holds a temperature-controlled water flow to maintain a constant body temperature. As an elegant development, relevant stomach and small intestinal dimensions are used and adapted to 6-month-old infant conditions. The small intestinal compartment is made of five tubular sections continuously squeezed during digestion in order to reproduce peristaltic and segmentation motions. The overall digestion lasts 248 min with the following sequence: oral phase (8 min), gastric phase (2h), intestinal phase (2h). The oral (esophagus), gastric and intestinal compartments remain static and maintained at pH 7, 3 and 6, respectively. The oral pH is adjusted according to *in vivo* data, the static gastric pH is chosen to represent mean data observed between the first months to second year of life as no data specific for 6 months infant was found. The intestinal pH used corresponds to adult value. The α-amylase used in the oral phase is added based on *in vivo* data in adults at a concentration of 75 U/mL. Every 10 min, 10 mL of the incubated food bolus are injected into the esophageal compartment, together with gastric fluids composed of electrolytes and pepsin (if tested foods contain proteins). Surprisingly, a high pepsin concentration was used in this study (1000 U/mL), compared to other child models and was based on data from adult models whereas *in vivo* data in child are available (Tavares et al., 2014). During the gastric phase, the stomach is hand squeezed every 15 min after sampling, which is not relevant compared to *in vivo* data. At the end of gastric phase, the pyloric

sphincter, opening and closing in a physiologically relevant manner (4 times per min in infant), allows the release in the intestinal compartment of a relevant chyme volume, depending on digested food. Gastric emptying is derived from a power exponential mathematical equation with parameters adapted to infant conditions and half-time emptying calculated according to food caloric density. During the intestinal phase, α-amylase, glucoamylase and porcine pancreatic lipase are added depending on the digested food. Another well-validated multi-compartmental model of the upper human gut is the TNO gastrointestinal system 1 (TIM-1), developed by Netherland Organization for Applied Scientific Research. This model has been first described in 1995 (Minekus et al., 1995) under adult digestive conditions and thereafter adapted to child ones, as related in several publications from TNO (Havenaar et al., 2013b, 2013a) and Université Clermont Auvergne in France (Blanquet et al., 2004 ; Roussel et al., 2016). TIM-1 contains four serial compartments reproducing the stomach and three parts of the small intestine, i.e. duodenum, jejunum and ileum. Each compartment is made of a glass jacket containing a flexible wall inside and connected to the others by peristaltic valves that regulate chyme transit. Body temperature and peristaltic mixing are achieved thanks to circulating heated water. Each compartment is characterized by specific pH kinetics (especially a gradual gastric pH drop), sequential delivery of secretions (gastric lipase and pepsin, porcine pancreatic juice and porcine bile salts), and transit time. This is the only model adapted to child digestion that reproduces passive absorption of small molecules and water. TIM-1 was adapted to simulate different age ranges and meal conditions: 6 months to 3 years old child ingesting a milk formula (Blanquet et al., 2004), 6 months to 2 years child drinking a glass of water (Roussel et al., 2016), 6 to 18 months child ingesting a light meal (Havenaar et al., 2013b), and term-neonates (0-1 month), infants (1-6 months) or toddlers (6-24 months) ingesting formula milk, formula milk or mixed fruit sauce and milk, juice and cereals, respectively (Havenaar et al., 2013a). In the seminal

study from Blanquet et al. (2004), half-time chyme delivery in both gastric (80 min *versus* 30 min -fast GI passage- or 70 min -slow GI passage-) and ileal (200 *versus* 160 min) compartments was increased in child compared to adult. While intestinal pH values of duodenal, jejunal and ileal compartments remained similar, gastric pH slope was less pronounced in child compared to adult (from 6.5 to 3.5 *versus* 4.5 to 1.5 -fast GI passage- or 5.5 to 1.7 -slow GI passage-). In this study, all digestive secretions were reduced in child compared to adult conditions: gastric pepsin (525 *versus* 600 U/mL) and lipase (30 *versus* 37.5 U/mL) activities, as well as porcine bile salts (1% *versus* 4% from 0-30 min and 2% from 30-360 min) and pancreatin (2 *versus* 7%) concentrations. Since absorption mechanisms seem already mature at birth, the same dialysis conditions were applied in the jejunal and ileal compartments in child conditions as in adult conditions. Recently, Roussel and collaborators (2016) adapted the TIM-1 to similar child age for the simulation of drinking a glass of water (instead of a milk formula). Half-time of chyme delivery was also increased both in gastric (20 min *versus* 15 min) and ileal compartments (190 min *versus* 150 min) compared to adult conditions. Gastric pH remained higher in child than in adult model, even if the pH set-up value was lower with water due to the absence of milk buffering effect. In this study, pepsin and lipase activities were not modified compared to adult condition, while pancreatic juice and porcine bile salts concentrations were reduced as previously described in Blanquet et al. (2004). In the publications from TNO (Havenaar et al., 2013b, 2013a), a special configuration of the TIM-1 was used (Tiny-TIM), where a single compartment is used to reproduce the child small intestine. As already described in many other child models, the main distinct parameter compared to adult was a less pronounced gastric pH drop, while maintaining the same intestinal pH (6.5). For 6-18 months child, the gastric pH drops from 6.5 to 4.5 within 150 min digestion *versus* 5.5 to 1.7 in adult (Havenaar et al., 2013b). This was further adjusted from 6.7 to either 4, 3.2 or 2.4 for neonate, infant and toddlers, respectively

(Havenaar et al., 2013a). The gastric emptying time was similar for neonate and infant (half time emptying of 60 min) but longer for toddler (70 min), with a total digestion time of 6h for the three kid categories. Digestive secretions were adapted to the amount of protein intake from food matrix and not the age (Havenaar et al., 2013b).

Taken together, available data indicate that scientists reached a consensus when developing *in vitro* models by setting-up a less pronounced gastric pH drop in child compared to adults, while maintaining the same intestinal pH. Developments that are more controversial are observed regarding GI secretions where broad range of concentrations and enzyme activities are used between *in vitro* models. Likewise, many discordances persist for gastric and intestinal transit times depending on studies. This discrepancy can be explained by the paucity of *in vivo* data in healthy neonates, infants and toddlers due to their invasive character. Also, the presence of the food matrix is poorly considered in most studies. The age simulated is varying widely between studies, with models set-up on age range rather than a precise age. Lastly, it is important to underline that none of these models reproduces the microbial component of the child intestinal environment, focusing mainly on digestive mechanical and physicochemical parameters.

3.3 Colon models

3.3.1 Static mono-compartmental models

In batch systems, fecal microbiota is diluted in a nutritive medium constantly stirred (estimated fecal concentration of 10% w/v) and cultured during 24 to 48h in temperature-controlled vessel maintained under anaerobiosis (by flushing with nitrogen and/or carbon dioxide). Upon inoculation, the batch remains static with no further medium input or output. Available batch models of the infant (average) colon were set up by using fecal samples from infants of 5 to 11 months in the model of Gamage' team from Macquarie University in Australia (Gamage et al., 2017), and 1 to 5 months by Ahlborn and colleagues from Grassland

Research Center in Australia (Ahlborn et al., 2020). Ahlborn's team uses pre-digested raw sheep and cow milk as nutritive medium according to the simple and static method proposed by Minekus (Minekus et al., 2014). The suspension is mixed with a cysteine solution to reduce the medium and baby feces diluted in phosphate buffer. The mixture is incubated at 37 °C with constant flushing with carbon dioxide and fermentation lasts up to 24h. Gamage and colleagues use predigested cereal products according to an *in vitro* digestion protocol adapted to infant conditions, but after perform fermentation under anaerobic conditions for a 48-h period without any adaptation of the basal medium simulating large intestinal conditions (Gamage et al., 2017).

3.3.2 Dynamic mono- or multi-compartmental models

Dynamic colon models rely on continuous or semi-continuous (and not batch) fermentation systems, with regular input of nutritive medium and regular output of fermentation medium, this allows to define for each bioreactor (for multi-compartmental models) a specific residence time that can be extrapolated to *in vivo* transit time. Most of the culture media used to simulate the composition of ileal effluents are derived from the initial one described by Macfarlane and colleagues from University of Reading in UK for adult colon models (Macfarlane et al., 1989). As for batch systems, bioreactors are temperature-controlled and maintained under anaerobiosis. Colonic pH is continuously monitored and controlled, and redox potential can be measured in real time in some of the models. Human colonic conditions can be reproduced by a single vessel or by multiple bioreactors in series representing different anatomical parts of the colon (ascending, transverse and descending colon). As for batch models, intermediate or end-products of microbial fermentation such as gas and SCFA can be measured in the fermentation media or atmospheric phase of bioreactors and microbiota composition is determined.

3.3.2.1 Dynamic Mono-compartmental models

The first dynamic colon model adapted to child conditions was the single stage culture system called Polyfermentor Intestinal Model (PolyFermS), set-up by Lacroix's team from ETH Zurich in Switzerland (Cinquin et al., 2004). In this study, the continuous *in vitro* model reproduces the infant colon from 4 to 6 months old. The nutritive medium was made according to Macfarlane's adult medium and adapted to infant digestion of a standard formula, considering infant digestibility indices. In particular, protein fraction was reduced, and whey proteins, starch, maltodextrin and lactose were added to replace complex fibers. Bile salts, which are less concentrated in the GI tract of infant, were also reduced compared to adult (0.05 g/L *versus* 0.4 g/L, respectively). Three different successive residence time and pH values were applied in order to reproduce the three colonic parts in a single bioreactor: 4h/5.7, 8h/6.2 and 12.5 h/6.8 (residence time/pH). Residence time and colonic pH were not modified compared to the adult model. Of note, an innovative microbiota immobilization technique on mixed gellan-xanthan gel beads was implemented to inoculate PolyFermS fermenters for mimicking planktonic and sessile cell growth. This immobilization procedure allows modeling infant gut microbiota even with a very low amount of fecal sample is available and ensures recovery and viability of dominant cultivable bacteria from infant feces such as *Bifidobacteria* (Cinquin et al., 2004; Pham et al., 2019).

A very close 6-month colonic model was developed by University of Agronomic Science and Veterinary Medicine in Romania, the mono-compartmented system of *in vitro* colonic simulation (GIS1), using the same operating parameters and medium composition as described by Cinquin (Vamanu et al., 2013). The only notable change is the addition of twenty 10-mm diameter glass balls to better simulate the peristalsis motion. Other publications from ETH Zurich in Switzerland reported further adaptations of the PolyFermS model. First, Dostal and colleagues simplified the model to simulate only the proximal colon

of 2.5 years old child with the increase of retention time to 8h at pH 5.7 (Dostal et al., 2015). In many studies, the selected retention time relies on a general rule stating that proximal colon transit time is estimated to be about one-third of the total colonic transit time. The infant adapted medium used was developed previously (Le Blay et al., 2009) and mainly relies on the reduction of biliary salts concentrations from 0.4 to 0.05 g/L (Macfarlane et al., 1998). Then, Pham and collaborators further adapted PolyFermS for 2-month-old formula-fed infant (Pham et al., 2019). Different pH values were tested in order to represent the proximal colonic pH of breast-fed (pH 5) or formula-fed infant (pH 7), as well as different retention times (5h or 10h). The nutritive medium was adapted to younger infant according to Cinquin's study, by removing maltodextrin, increasing lactose (6.4 *versus* 5 g/L) and whey proteins (8.1 *versus* 7.2 g/L) and decreasing casein concentrations (0.5 *versus* 1.2 g/L) (Cinquin et al., 2004). Lastly, Doo and collaborators again extended potentialities of the proximal colon PolyFermS for 6-month-old formula-fed infant (pH 6, retention time 7.4 h) and 8-month-old African infant living in poor environmental conditions and fed with a mixed diet (pH 5.8, retention time 6.1h) (Doo et al., 2017).

The Copenhagen MiniGut (CoMiniGut), developed by University of Copenhagen in Denmark, initially aims to reproduce the adult colon, and was further used to mimic 6-month infant conditions (Wiese et al., 2018). The model, inoculated with frozen stool, is composed of five small vessels in parallel (final volume of 5 mL), stirred, oxygen-free and pH controlled. Notably, except the use of infant stool, none of the operating parameters was adapted to a 6-month infant physiology. Besides, the microbiota profiles reported in this work suggest that the system is not well- controlled (maybe due to very small reactor volumes) and microbiota composition is abnormal for infant with dominance of Proteobacteria, low Firmicutes and almost no Actinobacteria.

3.3.2.2 Dynamic Multi-compartmental models

ETH Zurich in Switzerland also provided an update of the previously-mentioned PolyFermS system by developing a multi-compartmental model of the child colon simulating the three parts of the colon using three serial reactors (Cinquin et al., 2006). All the parameters were adapted to 6-months infant based on *in vivo* data. The total mean retention time is set at 13h and fixed in each compartment at 4, 5 and 4h, respectively. pH is only controlled in the two first reactors and set at 5.9 (proximal) and 6.2 (transverse), and naturally stabilized between 6.6-6.7 in the last reactor (distal). A similar system was developed by University of Agronomic Science and Veterinary Medicine, the multi-compartmental GIS2, an adaptation of the GIS1 composed by three reactors in series (Vamanu et al., 2015).

The EnteroMix, initially developed by Danisco in Finland for adult studies (Mäkivuokko et al., 2005) was used for infant gut research by inoculating the model with infant stools under 1 year old (Salli et al., 2019). To generate enough fecal inoculum, infant stools produced within a week were frozen, pooled, diluted in the nutritive medium, and amplified prior to inoculation. Anymore, none of the other parameters has been adapted to the specific infant colon conditions. This complex model reproduces from the ascending colon to the sigmoid/rectum area, with four glass vessels and ensures for each of them a control of pH (5.5, 6.0, 6.5 and 7.0, in the ascending, transverse, descending and sigmoid /rectum parts, respectively). Each vessel is inoculated with the amplified fecal inoculum and after 3h, the fermentation begins with the addition of 3 mL of nutritive medium to the first vessel. Every 3-hours, 3 mL are transferred from one vessel to the following one. Finally, 12h later, 3 mL are discarded from the last compartment. The entire experiment lasts 48 hours.

Altogether, compared to batch models, dynamic semi-continuous and continuous *in vitro* models of the child colon allow a better simulation of *in vivo* conditions, even if they require more trained manipulators and are more expensive and time-consuming. Adaptation of critical

parameters, such as pH and retention time, that widely influence gut microbiota composition and activity, is a key feature of these models (Duncan et al., 2009; Tottey et al., 2017). Unfortunately, in some of the current developments, these parameters have not been adapted to specific child colon conditions, adult parameters remaining the standard. This probably results from paucity of *in vivo* data or high individual variability, especially when all differentiated colon compartments are considered.

3.4 Whole gastro-intestinal models

Currently, the only available *in vitro* model reproducing the whole human gut is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), developed by Ghent University in Belgium (Molly et al., 1993). This set-up was adapted from 4 to 18-month child conditions in the Baby SHIME (De Boever et al., 2001). Baby SHIME is a semi-continuous model, composed of six temperature-controlled vessels reproducing the stomach, duodenum, jejunum/ileum and ascending, transverse and descending parts of the colon. The colon compartments are inoculated with a pooled of three feces collected from 3 to 12 months infants. pH values are not adapted to child conditions but only maintained between fixed limits ranging for stomach from 3.8 to 4.1 and for small intestine from 5.5 and 6, as previously set for adult conditions (Molly et al., 1993). The pH values of ascending (between 5.5 and 6.0) and transversal colon (between 6.0 and 6.3) compartments were also set up based on adult data, while the last vessel corresponding to the descending colon was maintained from 6.3 to 6.5 based on 10-18 months infant stool pH. For each GI compartment, the transit time is set at 3, 3, 2, 8, 12 and 8h, respectively, as inferred from infant whole gut transit time reported in *in vivo* studies on 6-week old infants (McClure and Newell, 1999) and 1 to 4 years toddlers (Walker and Walker, 1985; Weaver and Steiner, 1984). The nutritive medium used, based on a milk formula intended for babies aged 4 to 18 months, is added to the first gastric vessel, three times per day. The SHIME system was also very recently adapted to mimic

toddler digestion (Bondue et al., 2020). Colonic compartments were inoculated with fecal samples collected from healthy formula-fed children aged between 1 and 2 years. Apart from using feces from toddlers, the model was further adapted by changing the composition of the nutritive medium even if there is no information about it in the publication. pH and retention time parameters were based on adult data.

4. *In vitro* child gut models: main application studies

Digestive *in vitro* systems represent a valuable alternative and complementary approach to *in vivo* studies in nutrition and health and their related fields of research. The selection of an *in vitro* model primarily depends on the research question to be answered (in particular interactions with physicochemical or microbial digestive parameters?), the targeted GI compartments and the number of tested compounds (**Figure 4**). Dynamic and multi-compartmental models allow an in-depth control and parameter investigations, under *in vitro* conditions closely related to the *in vivo* situation, but are not always best suited, e.g. for screening studies, as there are heavy to operate and let a low number of independent repetitions. This section summarizes the main applications of child *in vitro* gut models in nutritional, pharmaceutical and microbiological studies (**Table 2, Figure 4**). Currently, there is no published work regarding the use of these models in toxicological studies, even if many scientific questions raised in this field of research could be addressed with such approaches and tools.

4.1 Nutritional studies

4.1.1 Nutrient digestibility

In vitro child models developed by Dupont's team at INRAE in France were used to evaluate milk protein or lipid digestibility. First, static models were used to compare the stability of food proteins toward infant (2 weeks to 3 months) and adult digestion. Results demonstrated

that bovine β-lactoglobulin was more degraded in the infant model, while β-casein and hen egg ovalbumin were more slowly digested under infant conditions compared to adult ones (Dupont et al., 2010). Digestive kinetics of a commercial infant formula was also investigated under 28 days old infant (Ménard et al., 2018) and adult static digestion protocols (Minekus et al., 2014). As observed *in vivo*, gastric proteolysis was slower in infant conditions compared to adult, while lipolysis remained similar between the two sub-populations. Then, the bi-compartmental model DIDGI® was further employed to evaluate protein hydrolysis kinetics from infant formula and exhibited a robust similarity with *in vivo* data derived from piglets (Ménard et al., 2014). Results reported an extensive and rapid casein hydrolysis while α-lactalbumin and β-lactoglobulin remained stable during gastric digestion. α-lactalbumin, β-lactoglobulin and whey proteins were rapidly hydrolyzed during intestinal digestion. Of note, those *in vitro* results were not compared to *in vivo* data in infant. Such *in vitro-in vivo* correlations were conducted in the Dynamic Gastric Model (DGM) gastric model where digestion of β-lactoglobulin and lactoferrin in terms of proteolysis and emulsion characteristics under infant conditions (birth to 6 months) were correlated with *in vivo* datasets (Shani Levi et al., 2013). Lastly, digestibility of different types of casein (transglutaminase cross-linked caseinate and native caseinate) from various food matrices was studied in the Tiny-TIM model adapted to simulate GI infant (6-18 months) conditions (Havenaar et al., 2013b). This study showed no difference in digestion efficiency for all types of caseins between adult and infant conditions, but highlighted the impact of food matrix on protein digestion rate in adults. This model was further operated several times to evaluate overall milk protein digestion from different infant formulas (Maathuis et al., 2017).

Very recently, Luo and collaborators used their static model of the infant (from birth to 6 months) gastric and intestinal phases to evaluate the impact of a surface-active component commonly used as emulsifier in infant food products (milk fat globule membrane polar lipids)

on lipid digestion (Luo et al., 2019). The authors analyzed the digestion process of different sizes of lipid droplets covered or not with this emulsifier. Results showed that even if fat droplets covered with milk fat globules (as emulsifier) are less digested during the beginning of gastric phase compared to those associated with caseins (control), they released a higher amount of free fatty acids later during digestion.

Some published works report the impact of infant formula sterilization on protein digestibility. The impact of glycation and cross-linking on protein breakdown and release of β-casomorphins was investigated *in vitro* using the infant (2 weeks - 3 months) static GI model developed by Cattaneo's team (Cattaneo et al., 2017). The authors revealed a clear hindered effect of sugar glycation (i.e. lactose and maltodextrins present in infant formula) occurring during sterilization on the digestion of caseins and whey proteins. Similarly, the impact of various sterilization methods (classical sterilization or high-temperature short-time treatment) on starch digestibility from different infant foods (rice starch mixed with water, rice cream with or without an aliquot of rice flour fermented by a strain of *Lactobacillus paracasei*) was studied in the dynamic multi-compartmental MIDA model, reproducing 6-month infant digestion (Passannanti et al., 2017). Results indicated a greater starch digestibility for rice cream with the fermented aliquot and a greater release of D-glucose from starch after the high-temperature short-time treatment compared to classical sterilization.

4.1.2 Impact of food nutrients on child gut microbiota

The PolyFermS colon model was applied to assess the impact of various prebiotic compounds found in baby formula on the gut microbiota of a 4-month old baby (Le Blay et al., 2010). The prebiotics tested were oligofructose, oligofructose-enriched inulin and high solubility inulin. All the prebiotic supplementations induced an increase in total SCFA production associated with an increase in *Bifidobacteria* and a decrease in *Bacteroides* and *Clostridia*. In another study, Wiese's team used the CoMiniGut colon model inoculated with fecal samples from 6-

month infant to evaluate the impact of different human milk oligosaccharides on gut microbiota composition and activity (Wiese et al., 2018). The strongest bifidogenic effect was obtained with 3-Fucosyllactose and a higher production of butyrate was measured following addition of 3-Sialyllactose or 6-Sialyllactose compared to 3-Fucosyllactose. In order to evaluate the impact of commercially available cereal products on infant gut microbiota, Gamage's team conducted an oral, gastric and small intestinal static digestion of the products prior to exposure to its static mono-compartmental colon model inoculated with 2-11 months infant feces (Gamage et al., 2017). Addition of digested cereal products led to changes in gut microbiota composition by increasing abundance of bacterial families related to fiber degradation but decreased *Enterobacteriaceae* abundance. A higher concentration of acetate was observed whatever the cereal product, while production of propionate and butyrate fluctuated according to the tested compounds.

Besides, PolyFermS model was also used to evaluate the effect of other food nutrients on infant gut microbiota. First, Doo and collaborators studied the impact of nucleotides and nucleosides commonly added to infant formula on gut microbiota (Doo et al., 2017). Such supplementation enhanced microbiota metabolic activity, as evidenced by an increase in total SCFA production, and modulated the expression of genes involved in the two major butyrate synthesis pathways, iron acquisition, nucleotide and sulfur metabolism, but also co-factor and vitamin biosynthesis. Amino acid user populations and some butyrate producers increased while the enteric pathogen *Salmonella* decreased. Second, Dostal and colleagues used PolyFermS to investigate the impact of iron availability on butyrate production by 2.5 years old child gut microbiota (Dostal et al., 2015). Results showed that while a low or moderate iron deficiency favored butyrate production, a strong deficiency induced a decrease in butyrate production, consistent with a loss in butyrate-producing bacteria compared to a normal iron status.

4.2 Pharmaceutical studies

The static immediate transfer digestion model was used to evaluate the performance of the furosemide drug in neonates and young infants (Klitgaard et al., 2017). The amount of furosemide solubilized in the aqueous phase during *in vitro* digestion was used to estimate the amount of drug available for absorption *in vivo*. The results pointed out an increase in drug solubilization with food intake (frequent feeding reproduced), while performance was not influenced by GI digestion of this food (addition or not of digestive enzymes). In addition, the drug dosage form (immediate-release tablet) did not affect furosemide solubilization as compared to a non-formulated powder. The dynamic bi-compartmental Tiny-TIM model was used under child conditions to evaluate the bio-accessibility of different dosage forms of two drugs (paracetamol and diclofenac) under realistic conditions including co-medication (with esomeprazole) and ingestion of food. Paracetamol availability for intestinal absorption was not influenced by the different GI conditions associated with the three-age groups (neonate, infant and toddlers), or by the tested dosage form, food matrix and co-medication. However, diclofenac had a greater bioaccessibility when administered with a food matrix than with water, but was not influenced by co-medication (Havenaar et al., 2013a). Lastly, the whole TIM-1 model was used under child or adult digestive conditions to evaluate the pharmacokinetics of paracetamol when administered under two oral dosage forms (powder or sustained-release tablet), with a glass of water or a complete breakfast (Blanquet et al., 2004). Paracetamol absorption was greater when ingested together with a standard breakfast compared to when co-ingested with water, and a slow GI transit associated with the complete meal digestion resulted in a delay in absorption. The authors also evidenced that paracetamol was more rapidly absorbed as a free powder form comparatively to the sustained-release tablet form. These *in vitro* data were in accordance with *in vivo* data, indicating that TIM-1 is a suitable tool for pharmacological studies in child.

. The silicone human nipple mimic model (Gerrard et al., 2013) was used to assess the impact of infant suction pulse rate, flow rate and fat milk content on the release from a tablet of the sulforhodamine B dye into the mother milk. Results highlighted an increase in the tested compound release with increased suction pulse rate occurring during breastfeeding, while milk fat content did not play a significant role. In addition, the release of sulforhodamine B from those tablets was tested in the tongue mimic system. The authors demonstrated an increase in compound release when the tongue strength, in particular the compression increased (Scheuerle et al., 2017).

4.3 Microbiological studies

The impact of the PROBAC product, containing both prebiotic (lactulose) and probiotics (*Lactobacillus paracasei* YR and *Enterococcus faecium* VL47 strains), on the 6-month infant gut microbiota was studied in the colonic GIS1 model (Vamanu et al., 2013). As expected, the administration of PROBAC induced an increase in *lactobacilli* and *bifidobacteria* as the products contain lactulose and stimulate lactase synthesis by those bacteria. This model was further used to evaluate the impact of lactic acid bacteria strains on 7-year old child gut microbiota (Moroeanu et al., 2015). *Lactobacillus rhamnosus* E4.2 and *Weissella paramesenteroides* FTa1 which synthetize more efficiently exopolysaccharides, had a better probiotic potential than other tested strains, as assessed by their positive influence on beneficial strains, their ability to reduce potentially pathogenic microorganisms and their effect on gut microbial activity. The multi-compartmental gastric and small intestinal TIM-1 model was used to evaluate the survival of lyophilized *Lactobacillus* LY/SA 1 strain under child and adult conditions, when administered in milk and water, respectively (Blanquet et al., 2004). In both gastric and ileal effluents, *Lactobacillus* LY/SA 1 survival rate was higher under child than adult digestive conditions. This result could be explained by a higher gastric pH and a lower bile salt concentration in child, but also by the potential protective effect of

milk (as compared to water). Such *in vitro* data are consistent with *in vivo* data obtained however with other *Lactobacillus* strains.

Apart from probiotics, *in vitro* child models have been applied to study pathogen survival and virulence gene expression, as well as their interactions with gut microbiota. Using TIM-1 model, Roussel and colleagues evaluated the survival and virulence of the foodborne pathogen *Enterohemorrhagic Escherichia coli* O157:H7 under 6-months to 2-years infant digestive conditions *versus* adults (Roussel et al., 2016). After GI passage, a significantly higher percentage of viable pathogenic cells was recovered in ileal effluents under child compared to adult set. Expression levels of virulence genes were increased by a 125-fold in infant *versus* adult condition, and O157:H7 Shiga-toxins, responsible for systemic complications in patients, were only detected in child ileal effluents. These results suggest that differences in digestive physicochemical parameters may already partially explain the highest susceptibility of child to Enterohemorrhagic *Escherichia coli* infection compared to adults. In another study, infection with *Salmonella enterica* serovar Typhimurium M557 in 4 to 6-month child proximal colon was simulated in the PolyFermS model. Amoxicillin treatment, a broad-spectrum antibiotic, was tested with two different concentrations, on pathogen survival and gut microbiota composition/activity (Le Blay et al., 2009). Addition of gel beads colonized with *Salmonella* led to a high and stable colonization of the strain in the bioreactor within 7 days, associated with an increase in *Enterobacteriaeceae*, *Clostridium coccoides-Eubacterium rectale* and *Atopobium* populations and a decrease in *Bifidobacteria*. SCFA production was slightly impacted by *Salmonella*, with an increase in butyrate concentration and butyrate producers' group during infection. Antibiotic administration led to a decrease in *Salmonella* concentrations but also impacted commensal bacteria, especially *Clostridium coccoides - Eubacterium rectale* decreased, while *Enterobacteriaceae* increased. The stable and high level of *Salmonella* serovar Typhimurium reached, as well as the impact of the

antibiotic assessed in this *in vitro* model, were correlated with previous *in vivo* reports (Le Blay et al., 2009).

5. *In vitro* models of the child gut: future developments and perspectives

5.1 Main scientific challenges and technological limitations to be addressed

5.1.1 Dealing with paucity of *in vivo* data and high individual variability

First, information regarding the complex development of digestive functions in children from birth to 3 years is still missing (**Figure 5**). This absence of detailed observations and mechanisms obviously hampers the development of fully reliable *in vitro* models related to pediatric digestion. Indeed, validation of *in vitro* models is always restricted to factors for which *in vivo* data are available. Even if major scientific and technical advances have already been investigated in this field during the past decades, current *in vitro* models still show some cognitive and methodological gaps. Especially, a large number of differences between models have been emphasized, such as highly heterogeneous enzyme or bile concentrations, pH and transit time, even if the same age period was applied. Part- but certainly not all- of these differences are likely due to different conceptualization, validation and/or applications of the models. The second main limitation is the targeted age, which is rather a period of several months/years than a specific age; even if it is now well acknowledged that several modifications may occur during this timeframe. . Facing *in vivo* data limitations, some parameters from many *in vitro* models are still set-up under adult rather than child conditions. *In vivo* extrapolation of *in vitro* results obtained under adult conditions can then be hazardous and may lead to overdosing or adverse side effects in child. Lastly, every model development has to face with high individual variability in terms of digestive parameters. Most of current models are based on average values but extreme conditions could be reproduced to consider for instance high/low metabolizers.

5.1.2 Considering the importance of the oral phase

To date, none of the available models of the oral phase has been adapted to progressive solid food chewing (only milk diet), nor incorporates saliva modifications in the first years of life. In addition, GI models, even the most sophisticated, only handle liquid foods and cannot incorporate solid particles even if children from 6 months to 1 year of age ingest the latter. There is therefore a crucial need to more accurately simulate the oral step of food breakdown by mastication and its role in the subsequent steps of digestion (Peyron et al., 2019). *In vitro* models of the chewing process already developed for adult, such as the Artificial Mastication Advanced Machine (AM_2) developed by Université Clermont Auvergne in France (Woda et al., 2010), the Chewing Robot set-up by Massey University in New Zealand (Sun, 2012) or the Dento-Munch by Bristol University in UK (Alemzadeh and Raabe, 2007), could be adapted to child conditions. These models reproduce both biomechanical and physicochemical aspects of the masticatory process. To go further, the ideal situation would be to combine these masticator models with advanced dynamic GI systems, such as the Engineered Stomach and Small Intestine (ESIN) from Université Clermont Auvergne, which has been specifically designed to handle both liquids and real-size food particles (Guerra et al., 2016).

5.1.3 Better reproducing lipid digestion

Most of current *in vitro* studies focus on the digestibility of milk protein fraction where distinct profiles between adult and infant are clearly described (Gan et al., 2018; Nguyen et al., 2015). On the contrary, lipid digestion has not been in-depth investigated during *in vitro* child digestion (Abrahamse et al., 2012; Poquet and Wooster, 2016). However, it is clearly known that the underdeveloped digestive tract of infants can influence their ability to digest and absorb lipids, whereas they represent a major source of energy and essential components for growth and brain/retina cells development. Between 3 and 6 months of age, compared to

adults, the digestive tract of infants exhibits lower concentrations of pancreatic triacylglycerol lipase, different lipolytic enzyme activities, lower concentrations of bile salts and different bile salt conjugation/speciation (Abrahamse et al., 2012; Poquet and Wooster, 2016). Simulating digestion of lipids then remains a major challenge, further increased by the inconsistency in lipase and bile salt concentrations used among reported *in vitro* studies. In addition, most *in vitro* models fail to adequately reproduce gastric lipolysis since they do not contain or make use of incorrect gastric lipases (e.g. fungal lipase). The source of gastric lipase commercially available is a growing concern and some companies are now trying to develop recombinant lipase or lipase from animal origin, like from rabbit (Bakala-N'Goma et al., 2015; Poquet and Wooster, 2016). In addition, most of *in vitro* studies use bile salts from bovine or porcine origin that are clearly different from human ones and evolution of bile salt conjugation and speciation with age is never considered.

5.1.4 Towards a better integration of host motility and absorption phenomena

Another main limitation of current *in vitro* child models is their inability to accurately mimic peristalsis, even if it is of great importance since dysregulations of GI motility (e.g. gastroparesis, dysphagia, gastro-esophageal reflux) are commonly observed in infants (Chumpitazi and Nurko, 2008). In most of *in vitro* child models, peristaltic mixing is reproduced *via* the use of magnetic stirrers, far from *in vivo* configuration. The only two child models that more closely reproduce peristaltic movements are the gastric DGM and the gastric and small intestinal TIM-1 system, through gentle mixing with pressurized water jackets. Efforts should be pursued to more accurately simulate mechanical deformations resulting from peristalsis, as already made for the stomach compartment in the Human Gastric Model (HGM) developed at Massey University in New Zealand, so far only set-up for adult condition (Ferrua and Singh, 2015). Nevertheless, technical improvement will be limited

since evaluation of motility in children is very challenging due to developmental maturation, as well as technical and cognitive issues in this population.

Regarding water and nutrient absorption, all *in vitro* child gut models are not designed to reproduce this phenomenon, except TIM-1 system that incorporates dialysis modules in the small intestinal compartments through addition of hollow fibers. This dialysis device allows mimicking passive absorption of water and small molecules (e.g fatty acids, oligo- and monosaccharides, small peptides and amino acids, drugs and chemicals), but also ensures bile reabsorption in the distal intestine, as occurring *in vivo* (Minekus et al., 1995). Such dialysis system has even been adapted to absorption of lipophilic compounds (Minekus et al., 2005) and could be easily extended to other *in vitro* models of the upper gut, since no specific adaptation is required for simulating child digestion, according to *in vivo* data (Bourlieu et al., 2014). To further reproduce absorption phenomenon and integrate active transport, *in vitro* child systems should be coupled with human intestinal epithelial cells, as previously done with TIM-1 and Caco-2 cells (Bahrami et al., 2011; Déat et al., 2009), or even organoids to move forward an increased complexity.

5.1.5 Challenge and technical improvement to mimic the gut microbiota

Most of the available systems integrating the host microbial component are modeling the colonic microbiota, through inoculation with fecal samples. Of note, there is a crucial lack of child *in vitro* models incorporating the buccal or gastric microbiota. One main advantage of *in vitro* child gut models is the possibility to easily consider microbiota associated with each segment of the small intestine and/or colon, and to follow changes in gut-derived metabolites, such as SCFA that are important modulators of host physiology (De Paepe et al., 2020; Poeker et al., 2018). In this endeavor, they represent a powerful platform for mechanistic studies on the role of infant gut microbiota without environmental disruptive factors and by discriminating host effects. However, some limitations have been raised from published

studies. First, a very low amount of fecal sample is usually available to inoculate bioreactors and technics allowing microbiota immobilization as developed by Lacroix's team in Switzerland can be an efficient answer (Cinquin et al., 2004). The treatment of fecal samples is also much debated as preservative method (e.g. freezing), and feces dilution or amplification may change microbial profiles and further impact metabolic activities. Besides, using pooled feces obviously removes individuality. Second, fecal samples are generally collected from child with age discrepancies, which can highly affect the results. There is also a crucial need to increase the number of volunteers to better take into account gut microbiota inter-individual variability and/or sex/gender differences. In most of available studies, *in vitro* colon models are inoculated with fecal samples from infant, but remained set-up with adult parameters (e.g. pH, transit time, composition of ileal effluents, bile salts), which could differentially shape gut microbiota structure and activity (Payne et al., 2012; Tottey et al., 2017). This limitation is still heightened by the paucity of *in vivo* data on child colonic microbiota composition (different from fecal microbiota), making difficult any *in vitro-in vivo* correlations. Third, if published *in vitro* studies consider longitudinal variations in child gut microbiota (from small intestine to distal parts of colon), none of them integrates radial changes from lumen to epithelium. Nonetheless, it is now acknowledged that in adults mucus-associated microbiota is clearly distinct from its luminal counterpart due to changes in nutrient availability and oxygen gradient (Etienne-Mesmin et al., 2019; Sauvaitre et al., 2021). If similar data are not available in child, we may speculate that such difference could also be found earlier in life (Donaldson et al., 2016; Rokhsefat et al., 2016). Integrating the mucosal compartment in various pediatric *in vitro* gut models would thus help to obtain a more realistic view of the spatial organization of child gut. This optimization can be performed based on work previously described in adult, especially in the Mucosal-SHIME (M-SHIME) from Ghent University in Belgium (Van den Abbeele et al., 2012) or other colon models

(Deschamps et al., 2020; Probert and Gibson, 2004). These models use mucin beads to create a mucosal microenvironment preferentially colonized by mucin-degraders and mucin-adherent bacteria. Lastly, to further investigate the mechanistic host-microbiome crosstalk, *in vitro* child gut models can been coupled to cell culture assays, as previously described (Bahrami et al., 2011; Chassaing et al., 2017a; Defois et al., 2018; Geirnaert et al., 2017; Marzorati et al., 2014a; Tovaglieri et al., 2019). Specific developments from Nickerson's group at Arizona State University consist in cell cultures in rotating wall vessels to mimic the microfluidics near to the mucosa (Nickerson et al., 2003). Even more complex units have been developed, such as the Host Microbiota Interaction Module (HMI) at Ghent University (Marzorati et al., 2014b) or recent microfluidic systems like HuMiX (Human-microbial crosstalk) (Shah et al., 2016) or gut-on-a-chip devices (Jalili-Firoozinezhad et al., 2019; Shin et al., 2019). Such models integrate oxygen gradient microenvironment and host cells, but can also reproduce relevant shear forces, as intestinal flow and peristalsis, that all impact gut microbiota.

5.2 Future applications

As their parameters can be adjusted in terms of target group (age-range), we can consider unlimited applications of *in vitro* child gut models (**Figure 5**). To date, these models have been mostly applied to nutritional applications, focusing on digestibility of infant milk formula. Next step would be to reproduce child digestive conditions when ingesting solid and not liquid foods. This will imply a complete adaptation of all physicochemical parameters that widely change depending on meal intake and food texture (Guerra et al., 2012; Singh et al., 2015). Apart from macronutrient digestibility, potentialities of *in vitro* models can be dedicated to the investigation of micronutrient bioaccessibility, such as vitamins A and D or calcium that are playing a key role during child growth. Adaptation of *in vitro* models to the digestion of solid food would also represent a powerful platform for pharmaceutical studies,

since food matrices widely impact oral formulation disintegration, and subsequent drug release and absorption, so called food-drug interactions (Blanquet et al., 2004; Bushra et al., 2011; Havenaar et al., 2013a; Schmidt and Dalhoff, 2002). Current applications in pharmaceutical studies are restricted to *in vitro* models of the upper gut. Another main challenge that could be addressed by child gut models is deciphering how pediatric drugs interact with gut microbiota, i.e. if drugs can be metabolized by resident microbiota and conversely if they can impact gut microbiota structure and/or metabolic activity (Lucafò et al., 2020; Swanson, 2015).

If *in vitro* systems are a relevant complement to *in vivo* studies in nutritional and pharmaceutical applications, they are becoming irreplaceable tools in microbiological and toxicological studies, when investigating pathogenic microorganisms or environmental/food contaminants. In these fields of research, *in vitro* digestion models are likely to become increasingly important for comprehensive health hazard and risk assessment in infant at-risk population, but also represent valuable instruments to develop and evaluate remediation strategies. Few microbiology studies have been yet performed using child *in vitro* models, opening up a large field of investigations. *In vitro* digestion models, and particularly dynamic and multi-compartmental systems, could then be advantageously used to assess the effect of successive conditions encountered by pathogenic microorganisms within the GI tract (including physicochemical factors and host microbes) on their survival and virulence. This may help scientists to unravel microbes' pathogenesis and particularly decipher whether differences in digestive physicochemical parameters or gut microbiota related to age conditions are crucial factors in the onset of disease, as previously shown by Roussel and colleagues (Roussel et al., 2016) for an *Escherichia coli* pathotype. Moreover, those *in vitro* models could provide an efficient platform to apply facets of infection therapeutics, such as antibiotics that are the most prescribed medications in neonatal and pediatric populations and

have long-term deleterious effects on their gut microbiome (Gibson et al., 2015). They would also be helpful in designing novel remediation strategies such as bacteriocins (Zihler et al., 2010), phages (Breitbart et al., 2008; Endersen et al., 2017) but also probiotics (Cordonnier et al., 2015; Etienne-Mesmin et al., 2011; Roussel et al., 2016; Thévenot et al., 2013; Vamanu et al., 2013), prebiotics (Sauvaitre et al., 2021) and their symbiotic combinations. Of note, like for pathogenic strains, multi-compartmental models are extremely useful to investigate probiotic survival and metabolic activity (especially bacteriocin production) in the child digestive tract, in a temporal-spatial manner (Chaikham et al., 2013; Cinquin et al., 2006; Cordonnier et al., 2015; Martinez et al., 2013; Molly et al., 1996; Rehman et al., 2012; Van den Abbeele et al., 2016; Zihler et al., 2010). Regarding toxicological studies, up to date, there is no published study on the fate of environmental pollutants (e.g bisphenol A, organic pollutants, heavy metals, emerging contaminants like micro and nanoplastics…) in child *in vitro* gut systems. Infant models can help in assessing the fate of such pollutants to predict the internal exposure and thereby contribute to a more accurate risk assessment (Vrijheid et al., 2016). Indeed, *in vitro* upper and lower GI systems would help investigating the interactions of food chemical contaminants with main physicochemical parameters of the child digestive tract (low pH, enzymes, bile…), as well as resident gut microbiota (so called toxicomicrobiomics) (Abdelsalam et al., 2020; Sharma et al., 2017; Tralau et al., 2015). They would thus give useful information on the fate, bioaccessibility and metabolization (if any, especially by gut microbiota) of those compounds in the child gut, when ingested with different food matrices, taking also into account the role of soil that is frequently swallowed by infants (Defois et al., 2018, 2017; Joly et al., 2013; Liu et al., 2018; Yin et al., 2016). Lastly, all the potentialities of child *in vitro* models applied to food pollutants may be extended to food additives, e.g. sweeteners, flavor enhancers, food coloring, preservatives or

emulsifiers (Cao et al., 2020; Chassaing et al., 2017b; Gerasimidis et al., 2020; Rinninella et al., 2019; Roca-Saavedra et al., 2018).

To date, all the available *in vitro* child models have been developed to simulate healthy conditions. Especially, digestive conditions of rich-country infants have been mostly mimicked, and simulating the digestive tract of children living in poor nutritional and sanitary conditions would be a first task (Doo et al., 2017; Toe et al., 2020). Another major scientific and technical challenge would be to optimize *in vitro* upper and lower GI models to reproduce child-diseased conditions, especially those associated with trouble in GI motility (such as gastroparesis, gastric reflux or pyloric stenosis) or gut microbiota perturbations (so called intestinal dysbiosis) described in necrotizing enterocolitis, obesity or mental disorders (Koleva et al., 2015; Moossavi and Azad, 2019; Underwood et al., 2020). In these two last situations, small intestinal and/or colonic models could be inoculated with feces collected from infant patients, and the main objective would be to maintain gut microbiota dysbiosis (considered as a typical feature of these pathologies) in the bioreactor for a sufficient period of time in order to test remediation strategies, such as probiotics.

Conclusion

Digestion is an essential but sophisticated process for human health widely evolving within the first years of life. This paper offers a complete and well-organized state of the art on child digestive physiology including interplay between morphological, physicochemical, mechanical and microbial features. The review also compiles and describes the main *in vitro* models simulating child digestion processes, from the simplest to the most sophisticated systems. It addresses to all scientists or industrials who aims to set up *in vitro* infant protocols or want to select the best models for developing or testing their compounds. The technical developments of child gut models are currently impeded by the paucity of *in vivo* data for

validation and the detrimental lack of consensus between studies on major GI parameters reproduced. The recent developments of non-invasive methods to follow these digestive parameters in humans open new avenues and may help in a near future to fill these scientific and technological gaps. Developing *in vitro* child models that are fully validated will enlarge even more their range of applications in food and pharmaceutical fields and boost the development of new child-specific products in food, nutrition and health.

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Figure 1. Food intake and texture exposure across childhood.

The three doughnut layers represent from outside to inside, the gradual exposure and transitioning to food textures, the type of food intake and the development of dental maturity, respectively, from birth up till 2 years of age. Food intake transitioning ends after 10 months of age, while exposure to complex textured food starts at that age.

Mo = months; Y = years.

Figure 2. Oral digestion development across childhood.

Key parameters of the oral phase are summarized according to the available literature data from birth up till 3 years of age. Lack of data are represented by "?".

Mo = months; Y = years.

Figure 3. Gastro-intestinal development across childhood.

Key parameters of the gastric, intestinal and colonic digestion/fermentation are summarized according to the available literature data from birth up until 3 years of age. Lack of data are represented by "?".

D = days; Dist = distal colon; Duo = duodenum; HCl = Hydrochloric acid; Jej = jejunum; Ile = ileum; Mo = months; Prox = proximal colon; Trans = transverse colon; Y = years.

Figure 4. Decipher the complexity levels of the *in vitro* childhood digestive models according to the parameters reproduced.

A. On top left, the pink gradient doughnut represents an "ideal model", as legend. In this model, all the key parameters of the digestion and fermentation are reproduced dynamically and gradually. The small doughnuts represent each class of *in vitro* models (listed according to first author publishing the original article related to the model, the model name is listed in brackets if available). The grey items mean that the parameters are not reproduced, while the pink items means that the parameter are reproduced, according to the legend on top left. **B.** Schematic representation of key *in vitro* models from static mono-compartmental to complete gastro-intestinal systems.

CoMiniGut = colon mini gut; CTDM = Continuous Transfer Digestion Model; DIDGI = gastro-intestinal dynamic digestion system; GIT = gastro-intestinal tract; GIS1= unicameral system of *in vitro* colonic simulation version 1; HNM = Human Nipple Mimic; ITDM = Immediate Transfer Digestion Model; MIDA = Model of an Infant Digestive Apparatus ; Poly FermS = Polyfermentor Intestinal Model; SHIME = Simulator of the Human Intestinal Microbial Ecosystem; TIM = TNO gastrointestinal system 1 ;TMS =Tongue Mimic System.

Figure 5. Challenges and future applications of the *in vitro* models of digestion across childhood.

The key challenges in the conception, innovation and/or improvement of the current *in vitro* digestion models are categorized. Four main future applications ranging from nutritional, pharmaceutical, toxicological to microbiological fields are described.

Table 1. Main characteristics of the current *in vitro* models mimicking the infant gastro intestinal tract.

Abs : absorption; Asc : ascending colon; Duo : duodenum; Des : descending colon; Jej : jenunum; Ile : ileum; SSF : Simulated Salivary Fluid; SGF : Simulated Gastric Fluid; SI : small intestine; SIF : Simulated Intestinal Fluid; Stom : stomach; RT : residence time; T°C : temperature; Trans : transverse colon; N/A : not applicable; (-) : information not available; X : not simulated in the model

MODELS	Age	pH / time (min) Transit time TT or Retention time RT (min or hours)								Food	Digestive secretions Nutritive medium	Abs	Microbiota	Mucosalphase	References
		Oralphase	Stomach	Small intestine			Large intestine								
				Duo	Jej	Ile	Asc	Trans	Des						
Static mono-compartmental models															
Upper GI tract models	*In vitro* infant digestion model (INRAE France)	2 weeks - 3 months	X	pH= 3 / 30 min	pH=6.5 / 30 min		X			Milk	Pepsin concentration reduced 8-fold* Bile salts concentration reduced 4-fold* Phosphatidylcholin, trypsin and chymotrypsin concentrations reduced 10-fold*	X	X	X	Dupont et al., 2010
				TT = N/A	TT = N/A										
		28 days	X	pH= 5.3 / 60 min	pH=6.6 / 60 min		X			Liquid infant formula + SGF + SIF	Pepsin concentration reduced 7-fold* Pepsin activity : 268 U/mL Bile salts concentration reduced 3-fold* Porcine pancreatin reduced 22-fold* Bovine bile reduced 3-fold*	X	X	X	Menard et al., 2018
				TT = N/A	TT = N/A										

	In vitro infant gastric digestion (Hunan Agricult Univ, China)	–	X	pH= 6.5 / 0–30 min pH= 6 / 30–60 min pH= 5.5 / 60–90 min pH= 5 / 90–120 min	pH=7 min / 120 min	X	X	Pepsin : 0.4 mg/mL Lipase : 0.8667 mg/mL Pancreatin : 1.6 mg/mL Bile salts : 5 mg/mL	X	X	X	Luo et al., 2019
				TT = N/A	TT = N/A							
	In Vitro Model Simulating Gastro-Intestinal Digestion (Univ Copenhagen, Denmark)	Neonates <2 months old	X	Fasted pH= 2.9 / 50 min Fed pH= 6.4 / 50 min	Fasted pH=7 / 120 min Fed pH=6.5 / 120min	X	X	Gastric lipase activity : 100 TBU/mL Pepsin activity : 63 U/mL/kg of BW Intestinal lipase activity : 50 TBU/mL Bile salts : 50 mM	X	X	X	Kamstrup et al., 2017
				TT = N/A	TT = N/A							
	ITDM - Immediate Transfer Digestion Model (Univ Copenhagen, Denmark)	0 - 2 months	X	Fasted pH= 2.8 / 50 min Fed pH= 6.4 / 50 min	Fasted pH=7 / 120 min Fed pH=6.5 /120min	X	Infant formula	<u>Fasted state</u> Gastric lipase activity : 100 TBU/mL Pepsin activity : 741.2 U/mL Pancreatic lipase : 250 TBU/mL Pancreatic extract Bile salts : 50 mM (sodium taurocholate) <u>Fed state</u> Gastric lipase activity : 17 TBU/mL Pepsin activity : 126 U/mL	X	X	X	Klitgaard et al., 2017
				TT = N/A	TT = N/A							

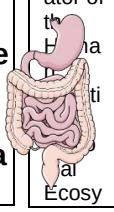
							Pancreatic lipase : 50 TBU/mL					
New pediatric two-stage dissolution model (Univ Greifswald, Germany)	–	X	pH= 1.8–4 / 30 min TT = N/A	pH=6.8 / 240 min TT = N/A	pH=6.8 / 480 min TT = N/A	Water Apple juice / sauce Yoghurt, Pudding SGF - SIF	SGF *sine* pepsin CarbSIF with 120 mM sodium chloride, 5 mM potassium chloride, and 15 mM sodium bicarbonate	X	X	X	Karkossa et al., 2017
Dynamic mono-compartmental models												
In vitro infant gastric digestion (Israel Inst Technol, Israel)	< 2 years	X	pH= 6.5 / 0-30 min pH= 6.5 / 30-150 min pH= 4.5 / 150-240 min pH= 3.5 / 240 min TT = N/A	X		–	Sodium chloride Hydrochloric acid Pepsin	X	X	X	Shani-Levi et al., 2013	
Dynamic bi-compartmental models												
DIDGI® - Dynamic Digester (INRAE, France)	–	X	pH (acidification curve) $T_{1/2}$ = 70 min β=1.23	pH=6.5 $T_{1/2}$ = 200 min β=2.2	X	Milk protein, dairy meat, vegtables, fruits, emulsionate	Pepsin : 1250 U/mL ; 0.25 mL/min Lipase 60 U/mL ; 0.25 mL/min Bile: bile solution (1 %) ; 0.5 mL/min Porcine pancreatin : pancreation solution (10%) ; 0.5 mL/min	X	X	X	Ménard et al. 2014	
CTDM - Continuous Transf	0 - 2 months	X	pH= 6.4 / 0-30 min pH=	Fasted pH=7 / 120 min Fed pH=6.5 / 120min	X	Infant formula	Gastric lipase activity : 17 TBU/mL	X	X	X	Klitgaard et al., 2017	

er Digestion Model (Univ Copenhagen, Denmark)			5.1 / 30-120 min				Pepsin activity : 126 U/mL Pancreatic lipase : 50 TBU/mL Fasted state Gastric lipase activity : 100 TBU/mL Pepsin activity : 741.2 U/mL Pancreatic lipase : 250 TBU/mL Pancreatic extract Bile salts : 50 mM (sodium taurocholate)				
			0-10 min: 0.84 mL/min 10-29 min: 0.25 mL/min 29-180 min: 0.14 mL/min								
Tiny TIM - TNO gastrointestinal system 1 (TNO, Netherlands)	6 - 18 months	Yes	pH 6.5 to 4.5 / 0-150 min	pH=6.5	X	Light meal	α-Amylase Gastric lipase Bile Pepsin Pancreatin	Passive (Jej + Ile)	X	X	Havenaar et al., 2013
			TT = N/A								
TIM pediatric - TNO gastrointestinal system 1 (TNO, Netherlands)	0 - 1 month	Yes	pH= 6.7 to 4 / 0-180 min pH 6.7 to 4 / 180-360 min	pH 6.5	X	For mula milk	Amylase Gastric lipase Pepsin Pancreatin	Passive (Jej + Ile)	X	X	Havenaar et al., 2013
			$T_{1/2}$ = 60min β=1.5	TT = N/A							
	1 - 6 months	Yes	pH= 6.7 to 3.2 / 0-180 min pH 3.8 to 3.2 / 180-	pH=6.5	X	For mula milk or mixed fruit sauce	Amylase Gastric lipase Pepsin Pancreatin	Passive (Jej + Ile)	X	X	

			360 min								
			$T_{1/2}$ = 60min β=1.5	TT = N/A							
	6 - 24 months	Yes	pH= 6.7 to 2.4 / 0-180 min pH 3.7 to 2.4 / 180-360 min	pH=6.5	X	Milk, juice and cereals	Amylase Gastric lipase Pepsin Pancreatin	Passive (Jej + Ile)	X	X	
			$T_{1/2}$ = 70min β=1.5	TT = N/A							
Dynamic multi-compartmental models											
MIDA - Model of an Infant Digestive Apparatus (Univ Naples, Italy)	6 months	pH=7 / 2 min	pH= 3 / 120 min	pH=6 / 120 min	X	Rice starch-based food, rice-cream based food SSF - SGF - SIF	α-amylase in SSF and SIF Pepsin in SGF Glucoamylase in SIF Pancreatic lipase in SIF	X	X	X	Passannanti et al., 2017
		TT = N/A	$T_{1/2}$ = 20-28 min	TT = N/A							
TIM1 - TNO gastrointestinal system 1 (TNO, Netherlands)	6 months - 3 yeas rs	X	pH= 6.5 / 0-30 min pH= 6.5 / 30-150 min pH= 4.5 / 150-240 min pH= 3.5 at 240 min	pH=6.5 / pH=6.8 / pH=6.8	X	Milk formula	Pepsin 525 U/ml Lipase 30 U/ml Pancreatin 2% Bile salts 1% Trypsin	Passive (Jej + Ile)	X	X	Blanquet et al., 2004
			$T_{1/2}$ = 70 min β=1.23	$T_{1/2}$ = 200 min β=2.2							
	6 months - 2 yeas rs	X	pH= 5.7 / 0-10 min pH= 5.3 / 10-	pH=6.4 / pH=6.9 / pH=7.2	X	Water	Pepsin 130 IU/min Lipase 5 IU/min Pancre	Passive (Jej + Ile)	X	X	Roussel et al., 2016

				20 min pH= 4.5 / 20-40 min pH= 3.2 / 40-60 min pH= 2 / 60-90 min						atic juice 3.5% Bile salts 2% Trypsin				
				$T_{1/2}$ = 20 min β=1.2	$T_{1/2}$ = 120 min β=1.7									
	Static mono-compartmental models														
	Gamage's model (Macquarie Univ, Australia)	2 - 11 months	Yes	pH= 3	pH=7				Cereals	α-amylase 150 U/mL Gastric pepsin 3125 U/mL Bile salt 2.5× 10^{-3}M Trypsin 10 U/mL, Chymotrypsin 2.5 U/mL Lipase 2000 U/mL, Colipase 4000 U/mL Amylase 20 U/mL Adult ileal effluents medium (from Macfarlane adult medium)	X	Child fecal sample (fresh) n=6	X	Gamage et al., 2017
				RT = N/A	RT = N/A										
Lower GI tract models	**Dynamic mono-compartmental models**														
	PolyFermS - Polyfermentor Intestinal Model (ESHM-ich	4 - 6 months	X	X	X	pH =5.7 RT = 4 hours	pH =6.2 RT = 8 hours	pH =6.8 RT = 12.5 hours	X	Derived from Macfarlane adult medium and adapted to infant digestion	X	Child fecal sample (fresh) n=4 + gellan-xant	Mucin in medium (porcine gastric typ	Cinquin et al. 2004	

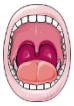
Switzerland)										han gel beads	e II)	
	2.5 years old	X	X	X	pH =5.7 RT = 8 hours	X	–	Derived from Macfarlane adult medium and adapted to infant digestion	X	Child fecal sample (fresh) n=1 + gellan-xanthan gel beads	Mucin medium (porcine gastric type III)	Dostal et al., 2015
	2 months	X	X	X	Various pH RT = 5 hours	X	–	From Cinquin infant medium (6 months old)	X	Child fecal sample (fresh) n=2 + gellan-xanthan gel beads	Mucin medium	Pham et al., 2019
GIS1 - *In Vitro* Colonic Fermentation Model (Univ Agronom Sci Vet Med, Romania)	6 months	X	X	X	pH =5.6-5.9 pH =6.2-6.5 pH =6.6-6.9 RT = not mentionned			Standard infant formula	Gastric juice (3 g/L pepsin + 3 g/L mucin) Bile salts (1, 2, 3, 4%) Pancreatin Derived from Macfarlane adult medium and adapted to infant digestion	X	Pool of child fecal samples (fresh) n=1 infant (several days of fecal collection)	Mucin medium	Vamanu et al., 2013
Dynamic multi-compartmental models												
CoMiniGut - Copenhagen MiniGut (Univ Copenhagen, Denmark)	6 months	X	X	X	pH =5.7-6 pH = 6-6.5 pH =6.5-6.9 RT = 8 hours			–	Sigma® colon medium for adult	X	Child fecal sample n=2 (frozen in 1M PBS /20 % glycerol	–	Wiese et al., 2018

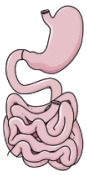
												(v/v)		
Three-stage PolyFermS-Polyfermentor Intestinal Model (ETH Zurich, Switzerland)	6 months	X	X	X	pH=5.9 RT = 4 hours	pH=6.2 RT = 5 hours	pH=6-6.7 RT = 4 hours	–	From Cinquin medium	X	Child fecal sample (fresh) n=2 + gellan-xanthan gel beads	Mucin medium (porcine gastric type II)	Cinquin et al., 2006
GIS2 - *In Vitro* Colonic Fermentation Model (Univ Agronom Sci Vet Med, Romania)	6 months	X	X	X	pH=5.5 RT = not mentionned	pH=6.2	pH=6.3	–	From Vamanu medium	X	Child fecal sample (fresh) n=1	Mucin medium (porcine gastric type II)	Vamanu et al., 2015
EnteroMIX® - Danisco (Finland)	< 1 yeaR	X	X	X	pH=5.5 RT = 3 hours	pH=6 RT = 3 hours	pH=6.5 (des) pH=7 (distal) RT = 3 hours (des) RT = 3 hours (distal)	–	From Macfarlane adult medium	X	Child fecal sample (fresh) n=2	Mucin medium (porcine gastric type III)	Mäkivuokko et al., 2005
Complete gastro-intestinal models														
Upper+lower GI tract 	Baby SHIME - Simulator of the Human Intestinal Ecosy	3 - 12 months	X	pH=3.8-4.1 / 180 min RT = N/A	pH=5.5-6 / 180 min RT = N/A	pH=5.5-6 / 120 min	pH=5.5-6 RT = 8 hours	pH=6-6.3 RT = 12 hours	pH=6.3-6.5 RT = 8 hours	–	Nutrilon Plus® medium	X	Pool of child fecal samples (fresh) n=19	Mucin medium	De Boever et al., 2001

models	stem (Ghent Univ, Belgium)	6 months	N/A	pH= 3.8-4.1 / 90 min	pH=6 /180 min	pH =5.5-6	pH =6-6.3	RT = 32 hours	X	–	From De Boever et al. 2001	X	Pool of child fecal samples (fresh) n=3	Mucin-agar beads (porcine gastric type III)	Van den Abbeele et al., 2019
	Toddler SHIME® - Simulator of the Human Intestinal Microbial Ecosystem (Univ Liege, Belgium)	1 - 2 years old	N/A	pH =3.8-4	pH=5.9-6.1	pH =5.4-5.6	pH =6-6.3	pH =6.3-6.5	RT = 72 hours	–	Medium PD-NM005 from Pro-Digest	X	Pool of child fecal samples (fresh) n=3	–	Bondue et al., 2020

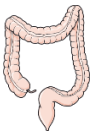
* when compared to adult condition

Table 2. Application studies of the different infant *in vitro* models in the fields of nutrition, pharmacology and microbiology.

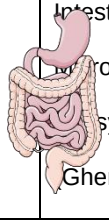
Models		**Applications**			**Aim of the study**	**Reference**
		Nutrition	Pharmacology	Microbiology		
Models of the oral phase 	HNM - Human nipple mimic (Univ Cambridge, UK)		+		Impact of milk composition on the release of sulforhodamine B dye into mother milk Impact of formulation (tablet) according to suction rate	Gerrard *et al*., 2013
	TMS - Tongue Mimic System (Univ Cambridge, UK)		+		Impact of milk composition on the release of sulforhodamine B dye into mother milk Impact of formulation (tablet) according to tongue compression / rotation	Scheuerle *et al*., 2017
Upper	**Static mono-**					

GI tract models 	**compartmental**					
	In vitro infant digestion model (INRAE, France)	+			Digestibility of milk proteins (β-lactoglobulin, β-casein and hen's egg ovalbumin)	Dupont *et al*., 2010
		+			Kinetics of lipolysis/proteolysis of commercial formula	Menard *et al*., 2018
	In vitro infant gastric digestion (Hunan Agricult Univ, China)	+			Impact of emulsifier in infant foods on lipid digestion	Luo *et al*., 2019 Bourlieu *et al*., 2014
	Cattaneo's model (Univ Milano, Italy)	+			Effect of glycation and cross-linking on protein breakdown from infant formula Release of β-casomorphins from formula	Cattaneo *et al*., 2017
	Pediatric two-stage dissolution (Univ Greifswald, Germany)		+		Screening drug release of a pediatric form (valproate-extended release formulation)	Karkossa *et al*., 2017
	In vitro digestion model (adaptation of Dupont et al., 2010)	+			Digestibility of beef puree	Dupont *et al*., 2010 Lee *et al*., 2019
	Dynamic mono-compartmental					
	In vitro infant gastric digestion (Israel Inst Technol, Israel)	+			Evaluation of proteolysis/emulsion of β-lactoglobulin and lactoferrin	Shani-Levi *et al*., 2013
	Dynamic bi-compartmental					
	DIDGI® -	+			Kinetics of protein hydrolysis from infant	Menard *et al*., 2014

	Dynamic Digester (INRAE, France)				formula Comparison with *in vivo* data in piglets	
	CTDM Continuous Transfer Digestion Model (Univ Copenhagen, Denmark)		+		Evaluation of oral performance of furosemide in infant and young children	Klitgaard *et al*., 2017
	Tiny TIM - TNO gastrointestinal system 1 (TNO, Netherlands)		+		Bioaccessibility of paracetamol and diclofenac with co-medication (esomeprazole) Impact of food matrices	Havenaar *et al*., 2013
		+			Kinetics of protein digestion and indispensable amino acids Impact of food matrices (goat, cow and human milk)	Maathuis *et al*., 2017
	Dynamic multi-compartmental					
	MIDA - Model of Infant Digestive Apparatus (Univ Naples, Italy)	+			Impact of various sterilization methods on starch digestibility from infant foods	Passannanti *et al*., 2017
	TIM1 - TNO gastrointestinal system 1 (TNO, Netherlands)		+		Pharmacokinetics of paracetamol (powder or sustained-release tablet) Impact of food matrices (glass water, complete breakfast) Impact of age conditions (adult *versus* infant)	Blanquet *et al*., 2004
				+	Survival of lyophilized Lactobacillus LY/SA 1	Blanquet *et*

					strain Impact of food matrices (glass water, milk) Impact of age conditions (adult *versus* infant)	*al*., 2004
		+			Evaluation of lipid digestion Impact of food matrices (human breast milk, Similac™ infant formulas)	Fondaco *et al*., 2014
				+	Survival and virulence of the foodborne pathogen Enterohemorrhagic *E. coli*	Roussel *et al.,* 2016
Lower GI tract models 	**Static mono-compartmental**					
	Gamage's model (Macquarie Univ, Australia)	+		+	Impact of commercially available cereal products (wheat, sorghum, rice or oats) on infant gut microbiota	Gamage *et al.,* 2017
	Batch system	+		+	Evaluation of human milk oligosaccharides on fermentation	Vester Boler *et al.,* 2013
	Dynamic mono-compartmental					
	PolyFermS - Polyfermentor Intestinal Model (ETH Zurich, Switzerland)	+		+	Impact of various prebiotics (oligofructose, oligofructose-enriched inulin and inulin) from formula on gut microbiota	Le Blay *et al*., 2010
		+		+	Impact of nucleotides/nucleosides from infant formula on gut microbiota	Doo *et al*., 2017
		+		+	Impact of iron availability on butyrate production by child gut microbiota	Dostal *et al*., 2015
				+	Impact of amoxicillin on Salmonella survival and gut microbiota composition/activity	Le Blay *et al* al., 2009
				+	Impact of pH and retention time on lactate metabolism and lactate-utilizing bacteria	Pham *et al*., 2019
	GIS1 - *In Vitro* Colonic Fermentation Model (Univ Agronom	+		+	Impact of symbiotic product (lactulose + *L. paracasei* + *E. faecium*) on infant gut microbiota	Vamanu *et al*., 2013
				+	Impact of lyophilized lactic acid bacteria strains on child gut microbiota	Moroeanu *et al.,* 2015

	Sci Vet Med, Romania)"					
	Dynamic multi-compartmental					
	3-stage PolyFermS - Polyfermentor Intestinal Model (ETH Zurich, Switzerland)			+	Interaction of *Salmonella* serovar Typhimurium with child gut microbiota Antagonistic properties of *E. coli* L1000 and *B. thermophilum* RBL67	Cinquin *et al.,* 2006 Zihler *et al.,* 2011
	CoMiniGut - Copenhagen MiniGut (Univ Copenhagen, Denmark)	+		+	Impact of different human milk oligosaccharides (3-Fucosyllactose, 3-Sialyllactose, 6-Sialyllactose and Fructooligosaccharide) on infant gut microbiota	Wiese *et al*., 2018
	GIS2 - *In Vitro* Colonic Fermentation Model (Univ Agronom Sci Vet Med, Romania)			+	Impact of two formulas containing probiotic on infant gut microbiota	Vamanu *et al*., 2014
	EnteroMIX® - Danisco (Finland)	+		+	Impact of human milk oligosaccharide (2'-fucosyllactose), galactooligosaccharides and lactose on infant gut microbiota	Salli *et al*., 2019
Upper+ lower GI tract models	**Complete gastro-intestinal**					
	Baby SHIME - Simulator of the Human Intestinal Microbial Ecosystem (Ghent Univ,	+		+	Impact of prebiotic (2'-fucosyllactose) from formula on infant gut microbiota	Van den Abbeele *et al*., 2019



	Belgium)					
	Toddler SHIME® - Simulator of the Human Intestinal Microbial Ecosystem (Univ Liege, Belgium)	+		+	Impact of symbiotic product (3′-sialyllactose + *B. crudilactis* on young child gut microbiota	Bondue *et al*. 2020

Highlights

- Gut anatomy, physiology and microbiota gradually mature from birth to 3 years old
- *In vitro* gut models are increasingly used as relevant complement to *in vivo* assays
- Child models reproduce all gut compartments with various levels of sophistication
- Technological developments are hampered by paucity and variability of *in vivo* data
- Nutrition/ health fields and industries would benefit from these new technologies

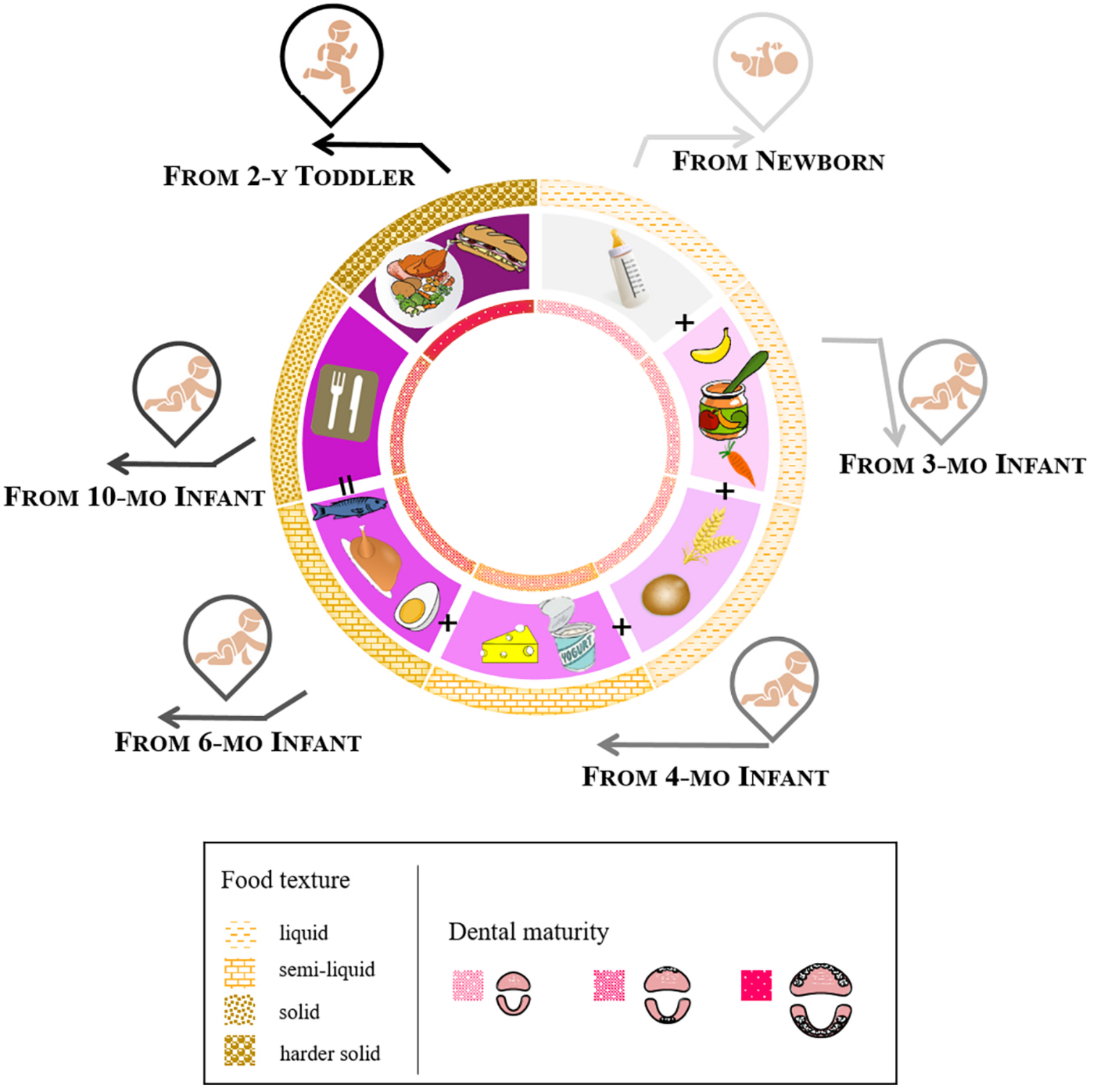

FROM 2-Y TODDLER
FROM NEWBORN
FROM 10-MO INFANT
FROM 3-MO INFANT
FROM 6-MO INFANT
FROM 4-MO INFANT
YOGURT
Food texture
liquid
semi-liquid
solid
harder solid
Dental maturity

Figure 1

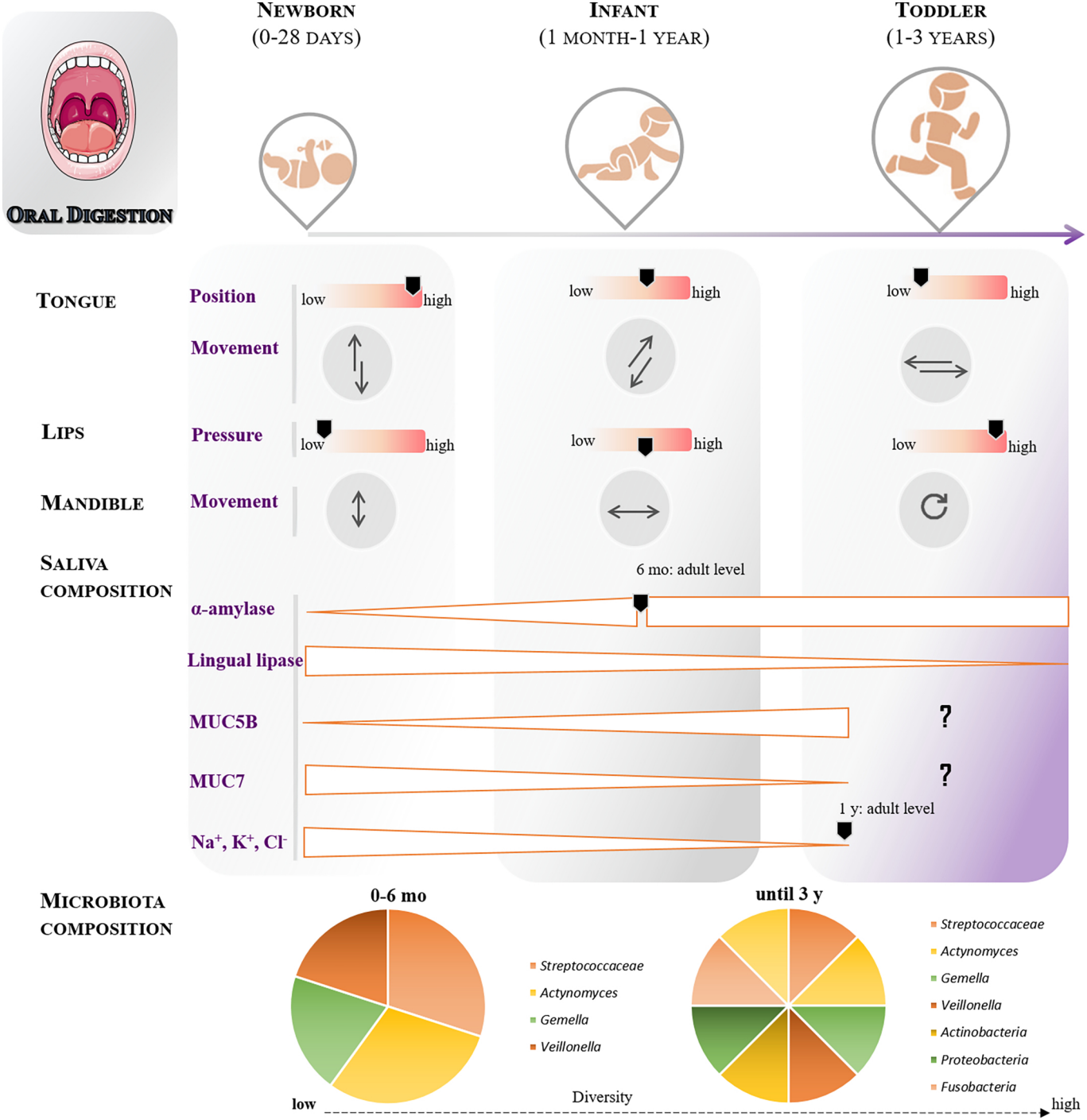

ORAL DIGESTION
NEWBORN
(0-28 DAYS)
INFANT
(1 MONTH-1 YEAR)
TODDLER
(1-3 YEARS)
TONGUE
Position
Movement
low
high
LIPS
Pressure
MANDIBLE
Movement
SALIVA COMPOSITION
6 mo: adult level
α-amylase
Lingual lipase
MUC5B
MUC7
Na+, K+, Cl-
?
1 y: adult level
MICROBIOTA COMPOSITION
0-6 mo
until 3 y
Streptococcaceae
Actynomyces
Gemella
Veillonella
Actinobacteria
Proteobacteria
Fusobacteria
Diversity
low
high

Figure 2

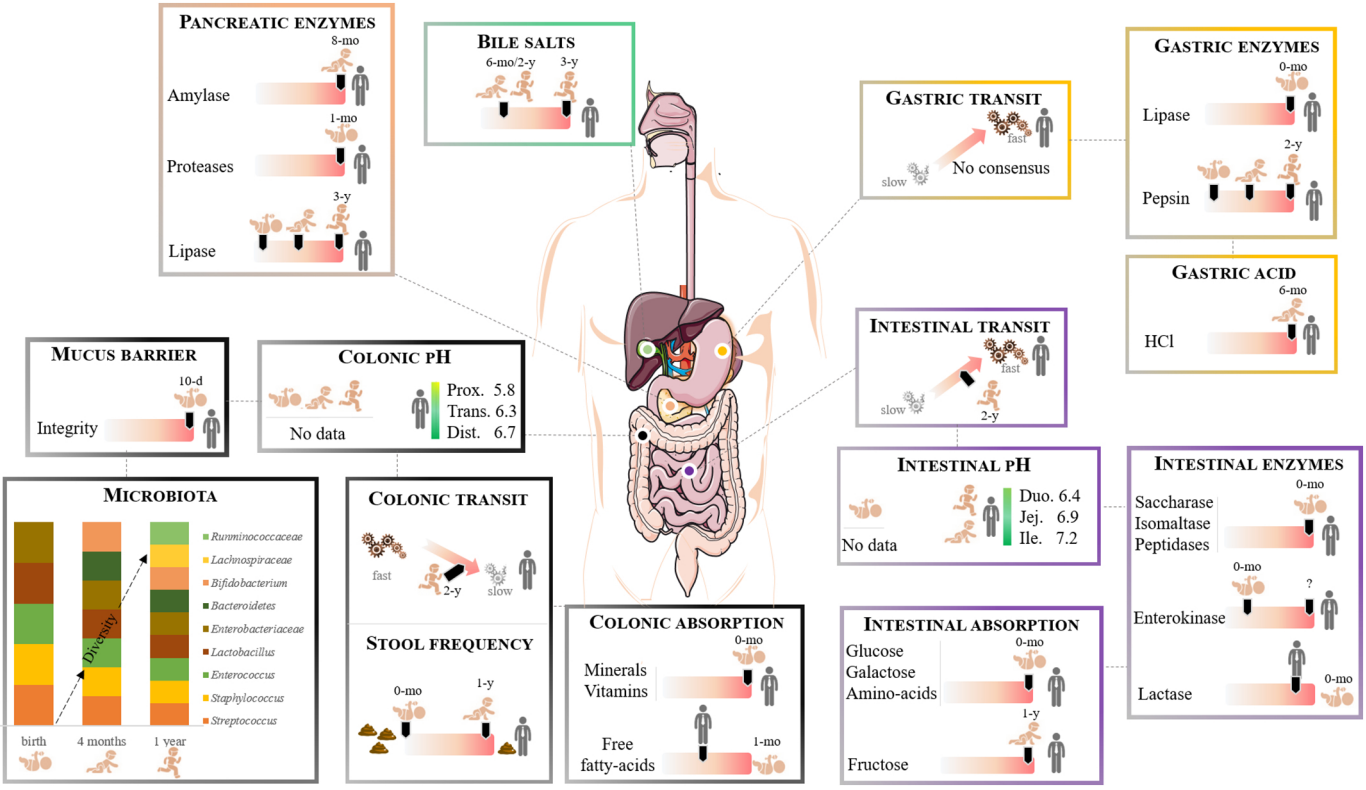

PANCREATIC ENZYMES
Amylase
8-mo
Proteases
1-mo
Lipase
3-y
BILE SALTS
6-mo/2-y
3-y
GASTRIC TRANSIT
slow
fast
No consensus
GASTRIC ENZYMES
Lipase
0-mo
Pepsin
2-y
GASTRIC ACID
HCl
6-mo
MUCUS BARRIER
Integrity
10-d
COLONIC pH
No data
Prox. 5.8
Trans. 6.3
Dist. 6.7
INTESTINAL TRANSIT
slow
fast
2-y
INTESTINAL pH
No data
Duo. 6.4
Jej. 6.9
Ile. 7.2
INTESTINAL ENZYMES
Saccharase
Isomaltase
Peptidases
0-mo
Enterokinase
0-mo
?
Lactase
0-mo
MICROBIOTA
Diversity
birth
4 months
1 year
Runminococcaceae
Lachnospiraceae
Bifidobacterium
Bacteroidetes
Enterobacteriaceae
Lactobacillus
Enterococcus
Staphylococcus
Streptococcus
COLONIC TRANSIT
fast
2-y
slow
STOOL FREQUENCY
0-mo
1-y
COLONIC ABSORPTION
Minerals
Vitamins
0-mo
Free
fatty-acids
1-mo
INTESTINAL ABSORPTION
Glucose
Galactose
Amino-acids
0-mo
Fructose
1-y

Figure 3

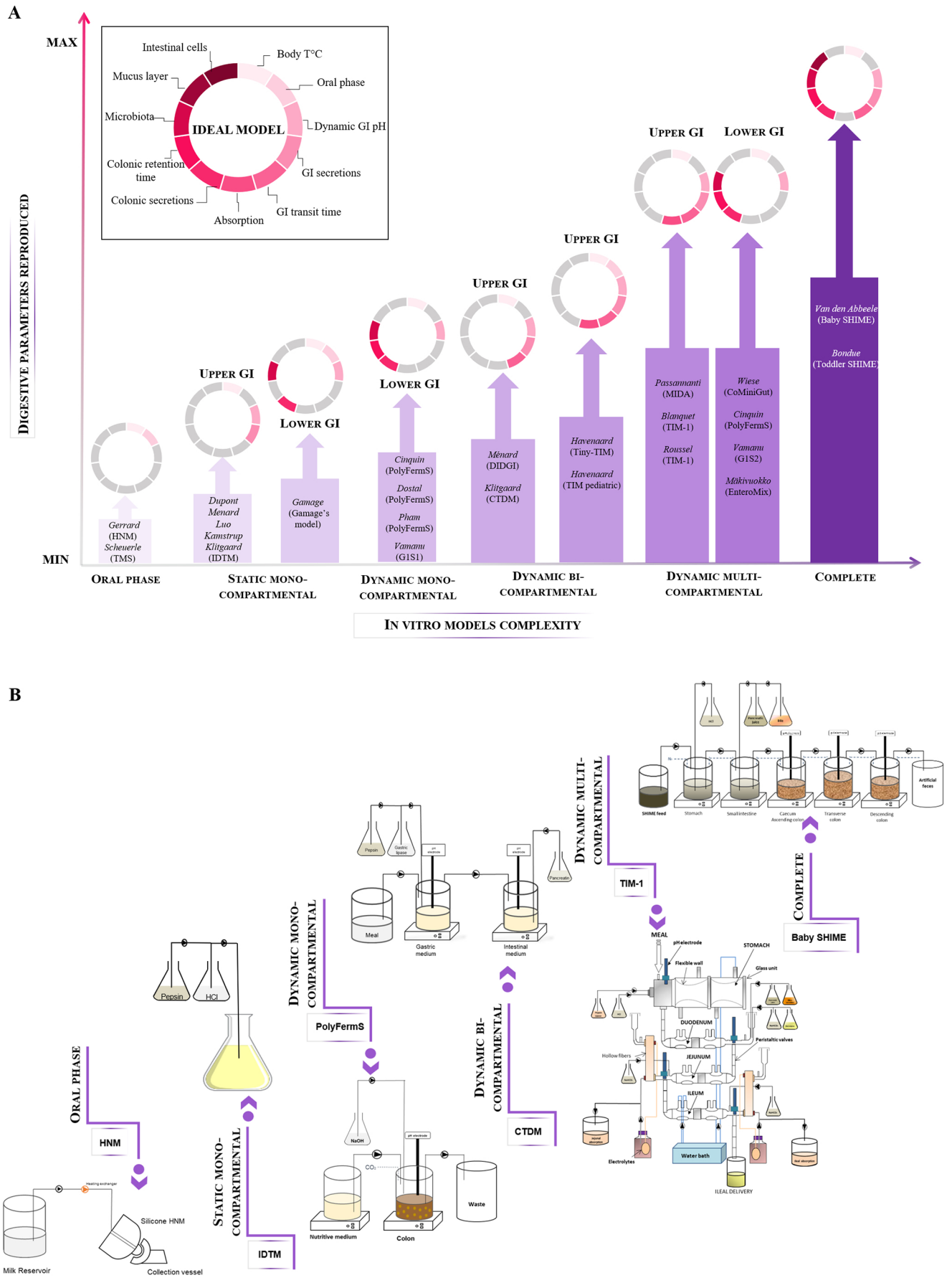
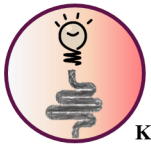

A
MAX
MIN
DIGESTIVE PARAMETERS REPRODUCED
IDEAL MODEL
Intestinal cells
Body T°C
Mucus layer
Oral phase
Microbiota
Dynamic GI pH
Colonic retention time
GI secretions
Colonic secretions
GI transit time
Absorption
Gerrard (HNM) Scheuerle (TMS)
Dupont Menard Luo Kamstrup Klitgaard (IDTM)
Gamage (Gamage's model)
Cinquin (PolyFermS) Dostal (PolyFermS) Pham (PolyFermS) Vamanu (G1S1)
Ménard (DIDGI) Klitgaard (CTDM)
Havenaard (Tiny-TIM) Havenaard (TIM pediatric)
Passamanti (MIDA) Blanquet (TIM-1) Roussel (TIM-1)
Wiese (CoMiniGut) Cinquin (PolyFermS) Vamanu (G1S2) Mäkivuokko (EnteroMix)
Van den Abbeele (Baby SHIME) Bondue (Toddler SHIME)
UPPER GI
LOWER GI
ORAL PHASE
STATIC MONO-COMPARTMENTAL
DYNAMIC MONO-COMPARTMENTAL
DYNAMIC BI-COMPARTMENTAL
DYNAMIC MULTI-COMPARTMENTAL
COMPLETE
IN VITRO MODELS COMPLEXITY
B
ORAL PHASE
HNM
Milk Reservoir
Silicone HNM
Collection vessel
STATIC MONO-COMPARTMENTAL
IDTM
Pepsin
HCl
DYNAMIC MONO-COMPARTMENTAL
PolyFermS
Nutritive medium
Colon
Waste
DYNAMIC BI-COMPARTMENTAL
CTDM
Meal
Gastric medium
Intestinal medium
DYNAMIC MULTI-COMPARTMENTAL
TIM-1
MEAL
STOMACH
DUODENUM
JEJUNUM
ILEUM
Water bath
Electrolytes
ILEAL DELIVERY
COMPLETE
Baby SHIME

Figure 4



IN VITRO MODELS OF DIGESTION ACROSS CHILDHOOD

KEY CHALLENGES

INFANT POPULATION DEFINITION
- Age (accurately define age-range)
- Sex
- Ethnic origin
- Food transition
- Nutritional status
- Gut-related diseases

PROTOCOL STANDARDIZATION
- Source of secretions (e.g. bovine, porcine origin)
- Fecal sample collection and treatment

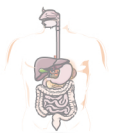
BIO ENGINEERING UPGRADE
- Food oral processing
- Lipid digestion
- Peristaltism
- Active transport
- Host crosstalk

(cells combined with digestion models)

MICROBIAL UPGRADE
- Longitudinal changes of gut microbiota
- Mucosal niche

GET BY
- *In vivo* data limitation
- Data heterogeneity
- *In vitro* limitations

(e.g. lack of nervous, endocrine, immune systems)



FUTURE APPLICATIONS

NUTRITIONAL FIELD
- Progressive solid food intake
- Macronutrient digestibility
- Micronutrient bioaccessibility (e.g. Vitamins A, D)

PHARMACEUTICAL FIELD
- Pediatric drug release and absorption
- Interaction/metabolization of drugs by gut microbiota

TOXICOLOGICAL FIELD
- Risk assessment of xenobiotics (fate, metabolization and bioavailability of :
  - Environmental/food contaminants (e.g. Bisphenol A, micro- and nanoplastics)
  - Food additives (e.g. sweeteners, emulsifiers)

MICROBIOLOGICAL FIELD
- Hazard and risk assessment of food-borne pathogens (survival and virulence)
- Infection therapeutic alternatives (e.g. probiotics, bacteriocins, phages)

SIMULATE DISEASE CONDITIONS
- GI motility trouble (gastroparesis)
- Microbiota perturbations (dysbiosis)
  - Necrotizing enterocolitis
  - Obesity
  - autism

Figure 5