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Samira Cássia de Oliveira

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AGRO CAMPUS OUEST

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Impact de la pasteurisation et de l'homogénéisation sur la digestion du lait maternel chez le nouveau-né : études *in vitro* et *in vivo*

Vincent RIOUX
Professeur AGROCAMPUS OUEST, UR AO Biochimie / *président*

André BRODKORB
Senior Scientist, Teagasc, Irlande / *rapporteur*

Jean-Louis BRESSON
Professeur, Pédiatre, Hôpital Necker Enfants Malades, Paris / *rapporteur*

Steven LE FEUNTEUN
Chargé de recherche, INRA AgroParisTech / *examinateur*

Valérie ALLGEYER
Directrice R&D, Lactalis, Retiers / *examinatrice*

Didier DUPONT
Directeur de Recherche, UMR INRA-AO STLO / *directeur de thèse*

Claire BOURLIEU
Chargée de recherche, UMR INRA-AO STLO / *co-encadrante de thèse*

Amélie DEGLAIRE
Maître de Conférences, UMR INRA-AO STLO / *co-encadrante de thèse*

Patrick PLADYS
Professeur, Pédiatre, Université de Rennes 1 et CHU de Rennes / *invité*

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Summary

Remerciements 1

Summary 5

Abbreviations 9

List of figures 11

Supplementary Figures 13

List of tables 15

Supplementary Table 15

General introduction, objectives and strategy 17

Introduction générale, objectifs et stratégie 22

Chapter 1 – Bibliographic review 28

1.1. Human milk: the ideal food in early nutrition 28

1.1.1. Specific composition and structure organization 28

1.1.2. Macronutrients 30

1.1.3. Micronutrients 36

1.1.4. Endogenous enzymes and the pre-digestion of human milk 36

1.1.5. Bioactivities beyond individual compounds 38

1.1.6. Human milk as a dynamic biologic fluid 40

1.2. How optimally nourish infants without access to breastfeeding? 43

1.2.1. First months feeding and health outcomes 43

1.2.2. Specificities of the preterm infant 44

1.2.3. Human milk fortification 45

1.2.4. Human milk banks 46

1.2.5. Infant formulas 48

1.3. Stabilizing human milk by physical treatments 51

1.3.1. Impact of Holder pasteurization and associated freeze-thaw cycles 51

1.3.2. Homogenization of pasteurized human milk 55

1.3.3. Alternative treatments to Holder Pasteurization 56

1.4. The developing digestive system 58

1.4.1. Newborn infant digestion 58

1.4.2. Gastric phase: a key step for infant digestion 60

1.4.3. Lipid digestion 62

1.4.4. Protein digestion 64

1.4.5. Emulsion disintegration 66

1.4.6. Studying the infant digestion 67

Chapter 2 – Gastrointestinal dynamic *in vitro* digestion of human milk in term and preterm infants: impact of Holder pasteurization 72

2.1. Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under *in vitro* dynamic term newborn digestion 74

2.1.1. Abstract 74

2.1.2. Introduction 74

2.1.3. Materials and methods 76

2.1.4. Results 81

2.1.5. Discussion 91

2.1.6. Conclusion 96

2.2. Impact of pasteurization of human milk on preterm newborn *in vitro* digestion: Gastrointestinal disintegration, lipolysis and proteolysis 97

2.2.1. Abstract 97

2.2.2. Introduction 97

2.2.3. Materials and methods 98

2.2.4. Results 101

2.2.5. Discussion 109

2.2.6. Conclusion 111

2.2.7. Supplementary material 112

2.3. Is the impact of pasteurization on HM digestion different in term and preterm newborns? 115

Chapter 3 – Impact of human milk pasteurization on gastric digestion in preterm infants: a randomized controlled trial 117

3.1. Abstract 119

3.2. Introduction 119

3.3. Methods 121

3.4. Results 128

3.5. Discussion 135

3.6. Supplementary material 138

Chapter 4 – Impact of homogenization of pasteurized human milk on gastric digestion in the preterm infant: a randomized controlled trial 140

4.1. Abstract 142

4.2. Introduction 143

4.3. Subjects and methods 144

4.4. Results 146

4.4. Discussion 154

4.5. Supplementary material 157

Chapter 5 – Specificity of gastric conditions during digestion of different types of milk: new *in vivo* data from preterm infants 159

5.1. Gastric lipase and other lipolytic activities in the preterm fed raw, pasteurized or pasteurized-homogenized human milk 161

5.1.1. Abstract 161

5.1.2. Introduction 161

5.1.3. Materials and methods 162

5.1.4. Results 164

5.1.5. Discussion 168

5.2. Physiological conditions during preterm gastric digestion and considerations for *in vitro* dynamic digestion simulation 170

5.2.1. Abstract 170

5.2.2. Introduction 170

5.2.3. Materials and methods 171

5.2.4. Results and discussion 171

5.2.5. Conclusion 175

Chapter 6 – Final considerations 177

6.1. General discussion 177

6.2. General conclusion and perspectives 182

6.2.1. Short-term perspectives 183

6.2.2. Long-term perspectives 183

References 187

Thesis outputs 217

Abstract 226

Résumé 227

Abbreviations

AA	Amino acid
AAP	American Academy of Pediatrics
ARA	Arachidonic acid
BSSL	Bile-salt stimulated lipase
CEH	Cholesterol ester hydrolase
CLSM	Confocal laser scanning microscopy
DG	Diglycerides
DHA	Docosahexaenoic acid
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology and Nutrition
FA	Fatty acid
FFA	Free fatty acid
FID	Flame ionization detector
GA	Gestational age
GC	Gas chromatography
HGL	Human gastric lipase
HM	Human milk
HMB	Human milk bank
HPL	Human pancreatic lipase
IF	Infant formula
LCPUFA	Long-chain polyunsaturated fatty acids
MCFA	Medium-chain fatty acids
MFG	Milk fat globule
MG	Monoglycerides
Mw	Molecular weight
NEC	Necrotizing enterocolitis
PHM	Pasteurized human milk
PHHM	Pasteurized-homogenized human milk
PLRP2	Pancreatic lipase related protein 2
PMSF	Phenylmethylsulfonyl fluoride
PUFA	Polyunsaturated fatty acids
RGE	Rabbit gastric extract
RHM	Raw human milk
SCFA	Short-chain fatty acids
SD	Standard deviation

SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
Sn	Stereospecific numbering
TG	Triglycerides
TLC	Thin layer chromatography
UNICEF	United Nations Children's Fund
VLBW	Very low birth weight
VLCFA	Very long-chain fatty acids
WHO	World Health Organization

List of figures

Figure 1. Global distribution of breastfeeding at 12 months of age. Data are from 153 countries between 1995 and 2013.. 19

Figure 2. Strategy for investigating the impact of physical treatments on the gastrointestinal digestion of human milk.. 21

Figure 3. Secretion process of human milk fat globules..... 30

Figure 4. A) Organization of lipids in human milk: structure of the milk fat globule and its trilayered membrane. B) Comparative fatty acids composition and regiodistribution in human and bovine milk triglycerides..... 31

Figure 5. Protein and lipid changes over lactation. 41

Figure 6. Hierarchy of the feeding choices for low birth weight infants. Source: Arnold (2006).... 45

Figure 7. Banked-human milk: steps from collection to distribution 48

Figure 8. Industrial process for infant formula fabrication and impact on the emulsion structure 49

Figure 9. Particle size distributions of human milk either raw, pasteurized or pasteurized-homogenized (RHM, PHM and PHHM, respectively). 55

Figure 10. Summary of the developing digestive physiology in the infant.. 59

Figure 11. Ontogeny, levels of activity or of mRNA expression (*) of the main digestive enzymes. 60

Figure 12. Gastric emptying rate fitted from experimental data using Elashoff model (A) and gastric pH decrease as reported in *in vivo* studies (B) 62

Figure 13. SDS-PAGE protein profiles of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion simulating term newborn conditions and the corresponding percentage of intact protein during gastric digestion as obtained by densitometry for lactoferrin (LF; panel C), serum albumin (SA; panel D), α-lactalbumin (α-Lac; panel E) and β-casein (β-CN; panel F)..... 82

Figure 14. Kinetics of lipolysis of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion simulating newborn term conditions..... 86

Figure 15. Acyl chains profiles initially esterified in human milk compared to free fatty acids released from RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion. 88

Figure 16. Particle size distributions (as determined by laser light scattering in SDS) and confocal laser scanning microscopy images (×60 zoom3) of undigested RHM and PHM. 88

Figure 17. Particle size distributions of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion..... 89

Figure 18. Confocal laser scanning microscopy images of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion. C) Decomposition of the images at 120 min of gastric digestion of RHM and PHM 91

Figure 19. Disintegration of preterm RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic preterm newborn digestion, and evolution of the associated size parameters (C). 102

Figure 20. Kinetics of lipolysis of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion simulating newborn preterm conditions. 104

Figure 21. SDS-PAGE protein profiles of preterm RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic preterm digestion. 107

Figure 22. Contribution of residual meal to the total gastric and intestinal contents after ingestion of term and preterm HM (histograms) and the corresponding ratio between enzyme activity and residual meal (lines) 115

Figure 23. Flow diagram of infants included in the study. The number of samples potentially collected per modality is indicated. 122

Figure 24. Microstructure of PHM and RHM before digestion. (A) Representative CLSM images (X60 zoom 3 and 6). Particle size distribution (B) and associated size parameters (C) were determined with the use of laser light scattering in water. 130

Figure 25. Changes with time in the gastric pH levels (A) and total volume (B) after ingestion of RHM and PHM. 130

Figure 26. Contribution of residual meal to total gastric content (A) and the rate of meal emptying (B) estimated with the use of soluble-phase taurine as a meal marker. 131

Figure 27. Changes in RHM and PHM structure during gastric digestion at 35 min (A), 60 min (B), and 90 min (C). 132

Figure 28. Kinetics of proteolysis of raw HM and pasteurized HM during gastric digestion: lactoferrin (A), serum albumin (B), α-lactalbumin (C), and β-casein (D). 133

Figure 29. Kinetics of RHM and PHM lipolysis during gastric digestion (A)and (B) FAs released from RHM and PHM at 90 min of gastric digestion. 134

Figure 30. Microstructure of undigested PHM and PHHM. 148

Figure 31. Changes with time in the gastric pH levels (A) and total gastric volume (B) after ingestion of PHM and PHHM. 149

Figure 32. A) Contribution of residual meal to the total gastric contents after ingestion of PHM and PHHM. The contribution of the gastric secretions is represented by the difference between the curves of total and residual meal volumes. B) Rate of meal emptying for PHM and PHHM 150

Figure 33. Disintegration of PHM and PHHM during gastric digestion.. 151

Figure 34. A) Kinetics of lipolysis of PHM and PHHM during gastric digestion. B) Acyl chains profiles initially esterified in human milk compared to free fatty acids released from PHM and PHHM at 90 min of gastric digestion 152

Figure 35. Kinetics of disappearance of major proteins in gastric aspirates after administration of PHM and PHHM. 153

Figure 36. Curve of specific activity of HPL, HGL and human cholesterol ester hydrolase (hCEH), considered as analogue to BSSL, against pH..... 162

Figure 37. Fasting gastric pH and total volume in preterm infants fed every three hours. 165

Figure 38. Fasting lipolytic activities in patients of group A (n= 12) and B (n = 8) measured at pH 4.5, 6 and 8..... 165

Figure 39. Fasting lipolytic activities at pH 4.5 and 6 against gastric volume A) and pH B). Data point represents triplicate determination by pH-stat..... 166

Figure 40. Postprandial lipolytic activities at pH 4.5 for group A, n = 4, and group B, n = 4. 166

Figure 41. A) Gastric pH decrease as reported in infants fed every three hours in previous and in the present *in vivo* studies, and those used for *in vitro* digestion simulation. B) Proposed fitting for gastric acidification by linear or polynomial curves. 173

Figure 42. Proportion of the ingested meal present in the stomach (A) and contribution of residual meal to the total gastric contents (B) at a given time after ingestion of raw, pasteurized or pasteurized-homogenized HM. 174

Figure 43. Proportion of samples presenting creaming (digested samples presenting higher concentration of fat than in the corresponding undigested HM) in groups A and B.. ... 175

Supplementary Figures

Supplementary Figure 1. Comparison of preterm and term undigested mature human milk: A) human milk collection conditions before pool; B) macronutrients, C) amino acids and D) fatty acids composition of pooled human milk. Pooled human milk structure before and after Holder pasteurization: E) confocal laser scanning microscopy images (x60 zoom 3) of preterm human milk before and after pasteurization. 113

Supplementary Figure 2. Evolution of the lipid classes composition (acyl chains, mol %) of preterm raw (A) and pasteurized human milk (B) during the gastrointestinal *in vitro* dynamic preterm digestion. 114

Supplementary Figure 3. Acyl chains profiles initially esterified in preterm human milk compared to free fatty acids released from raw (RHM) and pasteurized human milk (PHM) during gastric (A) and intestinal (B) *in vitro* dynamic preterm digestion. 114

Supplementary Figure 4. Rate of meal emptying for RHM and PHM, estimated using taurine (soluble phase) as a meal marker. 138

Supplementary Figure 5. Kinetics of decrease of major proteins during digestion of RHM and PHM. A: lactoferrin; B: serum albumin; C: α-lactalbumin; D: β-casein. 139

Supplementary Figure 6. Flow diagram of infants included in the study. The number of samples potentially collected per modality is indicated inetics of proteolysis of PHM and PHHM during gastric digestion. 158

Supplementary Figure 7. Kinetics of proteolysis of PHM and PHHM during gastric digestion.....**Error! Bookmark not defined.**

List of tables

Table 1. Indicative average composition of human and bovine milk per 100 mL..... 29
Table 2. Protein composition of term mature human milk..... 33
Table 3. Effects of Holder pasteurization on HM components 52
Table 4. Gastrointestinal conditions for *in vitro* dynamic digestion of human milk simulating term condition.....78
Table 5. Amino acid composition of human milk before *in vitro* digestion 83
Table 6. Amino acid released from RHM and PHM during intestinal *in vitro* dynamic digestion simulating term newborn conditions 85
Table 7. Gastrointestinal parameters for *in vitro* dynamic digestion of human milk simulating preterm conditions 100
Table 8. Fatty acid released from RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic preterm digestion..... 105
Table 9. Amino acids released from RHM and PHM during intestinal *in vitro* dynamic preterm digestion 108
Table 10. Background characteristics of infants (6 males, 6 females) who completed 6 days of follow-up..... 128
Table 11. Macronutrient composition and FA distribution of RHM and PHM..... 129
Table 12. Background characteristics of infants who completed 6 days of follow-up (n = 8) 146
Table 13. Macronutrients composition and fatty acids distribution of pasteurized and homogenized-pasteurized human milk 147
Table 14. Comparison of the values of lipase activity determined in fasting state in gastric aspirates of healthy full-term or preterm infants in the present and previous studies. Adapted from Bourlieu et al. (2014)..... 167
Table 15. The Holder pasteurization of HM impacts its gastrointestinal digestion (term and preterm stages)..... 179
Table 16. The ultrasonic homogenization of pasteurized HM impacts its gastric digestion (*in vivo* preterm stage) 180
Table 17. Impact of physical treatments on the chyme characteristics during the digestion of HM 180

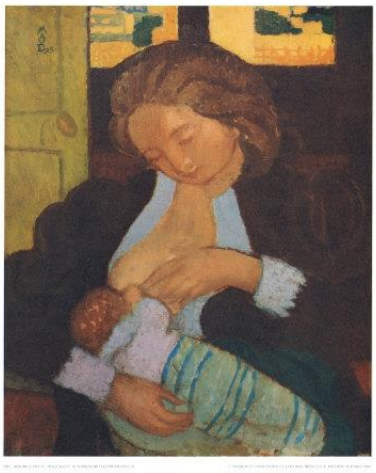
Supplementary Table

Supplementary Table 1. Amino acids composition of undigested preterm human milk..... 112

General introduction, objectives and strategy

"In the pure milk flows shed by my mom,
Not even a drop of bitterness was mingled"

Summer days, Marceline Desbordes-Valmore (1786 - 1859)



Maternity with lace cuffs (1895),
Maurice Denis (1870 - 1943),
Museum of Fine Arts, Rennes, France.

The painting and the poem excerpt, both from Dabadie's book (2013), describe the practice of breastfeeding over the nineteenth century. Such a positive perception about breastfeeding has been recorded over thousands of years among many peoples and civilizations. This is not surprising, since breastfeeding is a physiological and natural practice among mammals. However, considering *Homo sapiens,* it is not only a 'physiological evidence' but also a reflected choice that depends on many factors, such as cultural and socio-economic factors (Dabadie, 2013). Besides, sometimes breastfeeding is impossible due to medical issues linked to the mother or to the baby. **Whatever the reason, breastfeeding is not always possible and the choice of the substitute is recognized to be delicate since it has to respect the digestive immaturity of the infant, a new comer into the extra-uterine life.** That is why infant feeding practices for replacing breastfeeding have changed over time, following technological advances, understanding of the nutritional needs of the infant, cultural beliefs and scientific evidences.

A lactating woman breastfeeding an infant from another woman is an old practice originally called wet- or crossing-nursing, already mentioned in registers dating back from 2250 years Before Christ. This practice was widespread, and, from the 13th to the 19th century, European women could even earn money as wet nurses (Castilho & Barros-Filho, 2014; Guo, 2014a). Then, the fear of disease transmission, alongside with the development of techniques such as pasteurization and sterilization, led to an increase in the bottle practice feeding which was mainly based on bovine milk, as well on the 'medicalized version' of wet nursing, *i.e.* donation of expressed human milk (HM) undergoing heat treatment to avoid infections (Guo, 2014a; Dabadie, 2013). The first human milk bank (HMB) was established in Vienna, Austria, in 1909, followed by several units mainly in USA. By the middle of the 19th century, commercial infant formulas (IF) were largely promoted by health professionals due

to a link with scientific development and hence good nutrition. Furthermore, due to the discovery of HIV and the fear of contamination through the HM donation, many HMB were closed (Guo, 2014a). Nowadays, HM is well established as the gold standard in neonatal nutrition, especially when it comes to preterm infants. **Exclusive breastfeeding is recommended for the first six months of life**, with continued breastfeeding after food diversification for up to two years or longer (WHO, 2001). **Advantages of breastfeeding as ideal nourishment for the healthy growth and development of young children are recognized by national and international organizations** around the world, such as the American Academy of Pediatrics (AAP), the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) (American Academy of Pediatrics, 2012; Agostoni et al., 2009; WHO, 2001; WHO & UNICEF, 1989). These institutions manage several programs and initiatives which aim at increasing the prevalence of breastfeeding, such as “Baby Friendly Hospital Initiative” (WHO & UNICEF, 1989) based on “Ten steps to successful breastfeeding” (Labbok, 2012; Perez-Escamilla, Martinez & Segura-Perez, 2016).

Recently, a meta-analysis from Victora et al. (2016) has evidenced several breastfeeding benefits. For instance, they have reported that children who are breastfed for longer periods have lower infectious morbidity and mortality, fewer dental malocclusions and higher intelligence than those who are breastfed for shorter periods, or not breastfed; this inequality persists until later in life. Concerning the mothers, breastfeeding can prevent breast cancer, improve birth spacing, and might reduce risk of diabetes and ovarian cancer. This meta-analysis has also reported that, globally, high-income countries have shorter breastfeeding duration than low and middle-income countries. However, even in low-income and middle-income countries, only 37% of infants younger than 6 months of age are exclusively breastfed. **Figure 1** shows the global distribution of infants receiving any breastfeeding at 12 months of life.

Preterm infants are more vulnerable than term ones due to the high susceptibility to infections, digestive immaturity, complications due to impaired respiration, poor body temperature regulation, among other factors (WHO, 2015). The high prevalence and costs of prematurity have been considered as a public health problem and have received priority attention with program actions established either to prevent preterm birth or to improve the health care of mothers and preterm infants (WHO, 2012). According to the report “Born too soon” (WHO, 2012), 15 million babies were born prematurely in the world in 2010 (*i.e.* 11% of livebirths before 37 weeks of gestation), and this rate is increasing every year. Frequently unable to breastfeed due to the undeveloped reflex of sucking and swallowing, these infants often need to be fed by a nasogastric tube (Lau, 2015).

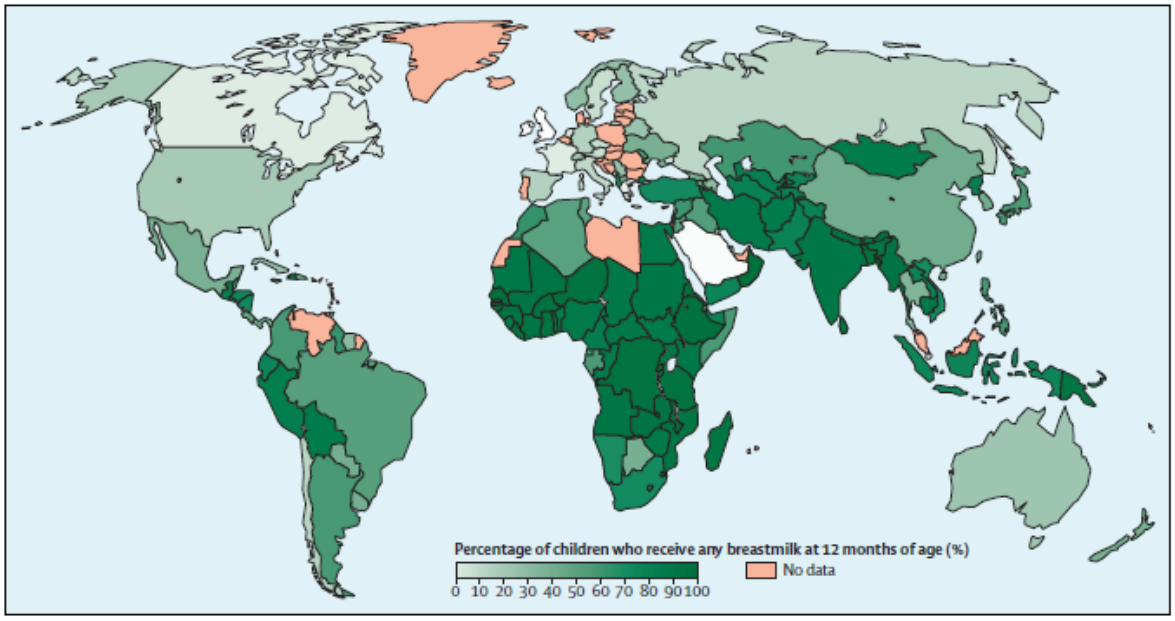


Figure 1. Global distribution of breastfeeding at 12 months of age. Data are from 153 countries between 1995 and 2013. Source: Victora et al. (2016).

Although in constant evolution, commercial IF cannot provide many of the bioactive components present in HM. Thus, expressed HM becomes the first choice for vulnerable infants who don't have access to breastfeeding. Indeed, HM contains several bioactive molecules which protect against infection, inflammation and contribute to immune and digestive maturation in early life (Donovan, 2016; Andreas, Kampmann, & Le-Doare, 2015; Labbok, Clark, & Goldman, 2004; Victora et al., 2016). Over the last decades, increasing scientific evidences of improved clinical outcomes of newborn infants fed HM instead of IF reinforced the practice of HM donation and encouraged the promotion of HMB implementation especially providing HM to vulnerable infants (Arslanoglu et al., 2013).

Nowadays, it is well established that different milk-based foods potentially given to newborn infants differ in terms of biochemical composition and structure, which can determine further nutritional and health consequences (Bourlieu & Michalski, 2015; Quigley & McGuire, 2014; Schanler, Lau, Hurst, & Smith, 2005). **Digestion, which is the intermediate step between the food and the organism, is recognized as a key step in determining nutrient pathways and hence nutritional and health outcomes.** However, very little is known about the fate of the different milk emulsions in the gastrointestinal tract of newborns. Some authors have reported differences depending on the gestational and postnatal age and the type of feeding, *i.e.* on the susceptibility to lipolysis, proteolysis, gastric emptying and secretion outputs (Roman et al., 2007; Armand et al., 1996; Cavell, 1981; Friedrikzon & Hernell, 1977). However, ethics and technical constraints limit the realization of clinical trials and, notably, the access to the intestinal phase of the digestion. With the advance in the proposition of *in vitro* models simulating digestion during the past few years, the number of studies

interested on better understanding the digestive process in infants has increased, but this still consists an ambitious objective.

Even though the benefits of breastfeeding were frequently extrapolated to expressed HM, in the present time it is well-known that **processing, storing and technological treatments modify the HM composition and may compromise its nutritional quality.** Among the technological treatments usually applied to HM for safety reasons, the Holder pasteurization is recommended by worldwide guidelines, despite side effects such as the level reduction or inactivation of endogenous lipases, immunoglobulins, lactoferrin and micronutrients (Peila et al., 2016; O'Connor, Ewaschuk, & Unger, 2015). In terms of physiological and nutritional impacts, pasteurization of HM may decrease lipid absorption and the mean weight gain in neonates (Andersson, Savman, Blackberg, & Hernell, 2007; Thomaz, Goncalves, & Martinez, 1999; Williamson, Finucane, Ellis, & Gamsu, 1978). Ultrasonic homogenization has been suggested to counteract the potential side effect of lipolysis reduction due to endogenous lipase inactivation by increasing the specific surface available for enzyme adsorption and hence fat digestibility, consequently improving growth of infants fed pasteurized-homogenized HM (Thomaz et al., 1999; Rayol, Martinez, Jorge, Goncalves, & Desai, 1993). However, there is no specific data on the impact of these treatments on the digestive behavior of HM.

Under this scenario, it seems important to answer the following question:

Does processing human milk impact its digestion in newborn infants?

In this context, **the main objective of this thesis was to answer this question by studying the impact of physical treatments (Holder pasteurization and ultrasonic homogenization) on the gastrointestinal digestion of HM in the newborn.** More specifically, this impact was evaluated in terms of kinetics of lipolysis, proteolysis and structural disintegration of the emulsions based on *in vitro* and *in vivo* studies.

Despite the richness and complexity of HM, we focused on the hydrolysis of these two major macronutrients, *i.e.* lipids and proteins, due to their importance for supporting infant growth (*e.g.* lipids that carry 40-50% of total HM calories) and their bioactivity (for proteins and their hydrolysis products). This choice was also justified by the recent interest on the structure of the HM emulsion, mainly defined by lipids and proteins, and its potential preprogramming effect (Oosting et al., 2014; Oosting et al., 2012).

The strategy used to accomplish this objective consisted in conducting *in vitro* and *in vivo* digestions of either raw, pasteurized or pasteurized-homogenized HM (respectively RHM, PHM and PHHM), as summarized in **Figure 2**.

Supported by an exhaustive literature review (Bourlieu et al., 2014), the dynamic digester parameters were determined for mimicking as closely as possible the gastric and intestinal digestive

conditions of preterm and term newborns, as presented in chapter 2. An *in vivo* study was then conducted on hospitalized preterm newborns at Rennes Hospital, as presented in chapters 3 and 4. The i*n vivo* study allowed us to evaluate some of the digestive parameters in fasting and postprandial states, as shown in chapter 5.

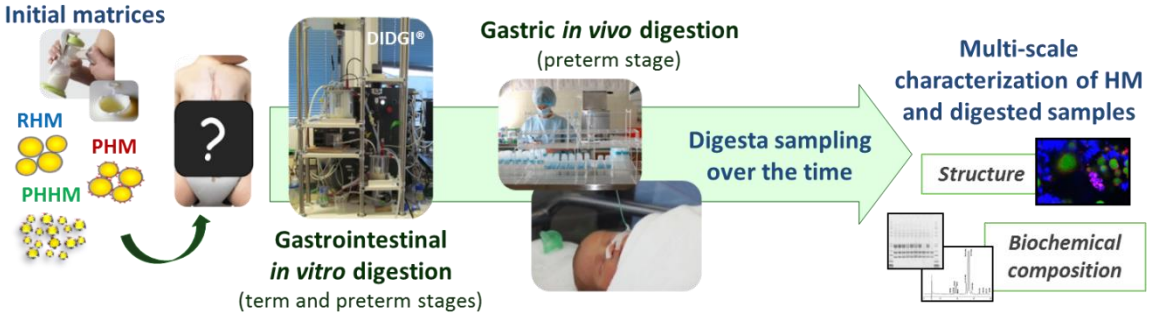


Figure 2. Strategy for investigating the impact of physical treatments on the gastrointestinal digestion of human milk. Raw, pasteurized and pasteurized-homogenized human milk are respectively RHM, PHM and PHHM.

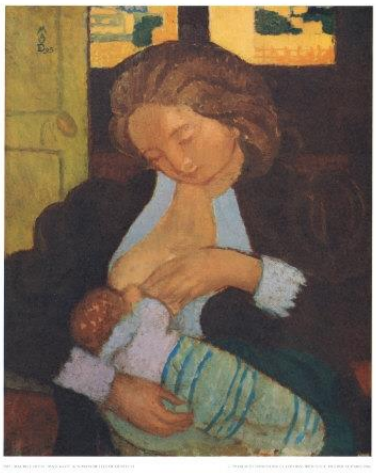
The present manuscript is organized as a series of six scientific papers, four of which are published and two are in preparation for submission. In addition, a first chapter includes a literature review and a last chapter presents the general discussion of the present work, an overall conclusion and perspectives.

This thesis was possible thanks to a network of collaborators that I would like to mention already at this stage, since their involvement was important for the success of this project. First of all, this thesis was integrated within a network of European researchers specialized in nutrition and digestion through the COST - INFOGEST FA 1005. On a collaborative point of view, the partnership with the University Hospital Center of Rennes allowed the implementation of the clinical trial, as well as having access to HM for *in vitro* digestion; it was possible thanks to the collaboration of Patrick Pladys, Amandine Bellanger, Emelyne Dirson and their teams. Some analysis concerning the characterization of lipolysis products were performed at the laboratory 'Enzymologie Interfaciale & Physiologie de la Lipolyse' (CNRS, Marseille) thanks to the collaboration with Frédéric Carrière, who also supplied enzymes for *in vitro* digestion. Analyses of HM macronutrients composition were assessed at the UMR PhAN (INRA, University of Nantes), thanks to the collaboration with Clair-Yves Boquien. Grants for a short-term scientific mission was provided by the COST project and allowed a one-month exchange with the 'Grupo Lípidos Departamento de Bioactividad y Análisis de los Alimentos' (CIAL, Madrid), a team with an expertise in lipidomics directed by Javier Fontecha.

Introduction générale, objectifs et stratégie

« C'est qu'aux flots de lait pur que me versait ma mère
Ne se mêlait alors pas une goutte amère »

Jours d'été, Marceline Desbordes-Valmore (1786 - 1859)



Maternité aux manchettes de dentelle (1895),
Maurice Denis (1870-1943),
Musée des Beaux-Arts, Rennes.

La peinture et l'extrait de poème, tels qu'affichés dans le livre de Dabadie (2013), illustrent la pratique de l'allaitement maternel au cours du XIXème siècle. Une telle perception positive de l'allaitement se retrouve dans de nombreux registres datant de milliers d'années et provenant de diverses civilisations. L'allaitement maternel étant une pratique physiologique et naturelle chez les mammifères, cela n'est pas surprenant. Néanmoins, en considérant l'*Homo sapiens*, l'allaitement n'est pas uniquement une « évidence physiologique », mais aussi un choix réfléchi qui dépend de nombreux facteurs, en particulier culturels et socio-économiques (Dabadie, 2013). L'allaitement peut aussi être rendu impossible pour des raisons médicales liées à la mère et/ou à l'enfant. Quelle que soit la raison, lorsque l'allaitement n'est pas possible, le choix du substitut est d'autant plus délicat qu'il doit respecter l'immaturité digestive du nouveau-né. Les pratiques d'alimentation du nourrisson ont changé au fil du temps, suivant les progrès technologiques, l'évolution de la compréhension des besoins nutritionnels de l'enfant, les croyances culturelles et des preuves scientifiques.

L'allaitement d'un nourrisson par une femme qui n'est pas sa mère est une pratique très ancienne, déjà mentionnée dans des registres datant de 2250 ans avant Jésus-Christ. Cette pratique était assez répandue, et, du XIIIème au XIXème siècle, les femmes européennes pouvaient même recevoir de l'argent en tant que nourrices (Castilho et Barros Filho, 2014 ; Guo, 2014a). Par la suite, la peur de la transmission de maladies, en parallèle du développement des techniques telles que la pasteurisation et la stérilisation, ont conduit à une augmentation de l'alimentation au biberon, principalement basée sur le lait de vache, mais aussi sur une « version médicalisée » des nourrices, à savoir le don de lait maternel exprimé et subissant un traitement thermique pour éviter les infections (Guo, 2014a ; Dabadie, 2013). Dans ce contexte, la première banque de lait maternel (lactarium) a été créée à Vienne, en Autriche, en 1909, et a été suivie par plusieurs unités principalement aux Etats-Unis. Vers le milieu du XIXème siècle, les professionnels de santé ont largement fait la promotion des

préparations commerciales pour nourrissons (c'est-à-dire formules infantiles), en raison d'un lien avec le développement scientifique et donc d'une bonne nutrition. De plus, en raison de la découverte du VIH et de la peur de la contamination par le don de lait maternel, plusieurs lactariums ont été fermés (Guo, 2014a).

Actuellement, le lait maternel est bien établi comme étant l'aliment référence pour la nutrition néonatale, ceci étant encore plus vrai pour les nouveau-nés prématurés. L'allaitement maternel exclusif est recommandé pendant les six premiers mois de vie, avec la poursuite de l'allaitement après diversification alimentaire jusqu'à deux ans ou plus (WHO, 2001). Les avantages de l'allaitement maternel pour la croissance et le développement des jeunes enfants en bonne santé sont reconnus par les organisations nationales et internationales à travers le monde, tels que l'Académie Américaine de Pédiatrie (AAP), la Société Européenne de Gastroentérologie, Hépatologie et Nutrition Pédiatriques (ESPGHAN), l'Organisation Mondiale de la Santé (OMS) et le Fonds des Nations Unies pour l'Enfance (UNICEF) (American Academy of Pediatrics, 2012; Agostoni et al., 2009; WHO, 2001; WHO & UNICEF, 1989). Ces institutions gèrent plusieurs programmes et initiatives qui visent à accroître la prévalence de l'allaitement, comme « Initiative hôpital ami des bébés » (WHO & UNICEF, 1989) basée sur les « Dix étapes pour un allaitement réussi » (Labbok, 2012 ; Perez-Escamilla, Martinez & Segura-Perez, 2016).

Récemment, une méta-analyse de Victora et al. (2016) a mis en évidence de nombreux avantages de l'allaitement maternel. Par exemple, ces auteurs ont rapporté que les enfants qui sont allaités plus longtemps ont de plus faibles taux de morbidités infectieuses et de mortalité, moins de malocclusions dentaires et un niveau d'intelligence plus élevé que ceux qui sont allaités pendant des périodes plus courtes ou non allaités ; cet écart persiste jusqu'à plus tard dans la vie. En ce qui concerne les mères, l'allaitement peut prévenir le cancer du sein, améliorer l'espacement des naissances, et pourrait réduire le risque de diabète et de cancer de l'ovaire. Cette méta-analyse a également signalé que, globalement, les pays à hauts revenus ont une durée d'allaitement plus courte que les pays à revenus faible et intermédiaire. Cependant, même dans ces derniers, seulement 37% des nourrissons de moins de 6 mois sont allaités exclusivement au sein. La **Figure 1** montre la distribution mondiale des nourrissons jusqu'à 12 mois de vie recevant l'allaitement maternel.

Les enfants prématurés sont plus vulnérables que ceux nés à terme en raison, entre autres, d'une forte sensibilité aux infections, immaturité digestive, complications respiratoires, mauvaise régulation de la température corporelle, etc. (WHO, 2015). La forte prévalence et les coûts liés à la prématurité sont considérés comme un problème de santé publique recevant une attention prioritaire, avec des actions établies soit pour prévenir l'accouchement prématuré, soit pour

améliorer les soins aux mères et aux nouveau-nés prématurés (WHO, 2012). D'après le rapport « Né trop tôt » (WHO, 2012), 15 millions de bébés étaient nés prématurément dans le monde en 2010 (11% des enfants nés vivants avant 37 semaines de gestation), taux qui augmente chaque année. Généralement incapables de se nourrir au sein en raison d'un réflexe de succion/déglutition peu développé, ces enfants ont besoin d'être nourris par une sonde nasogastrique (Lau, 2015).

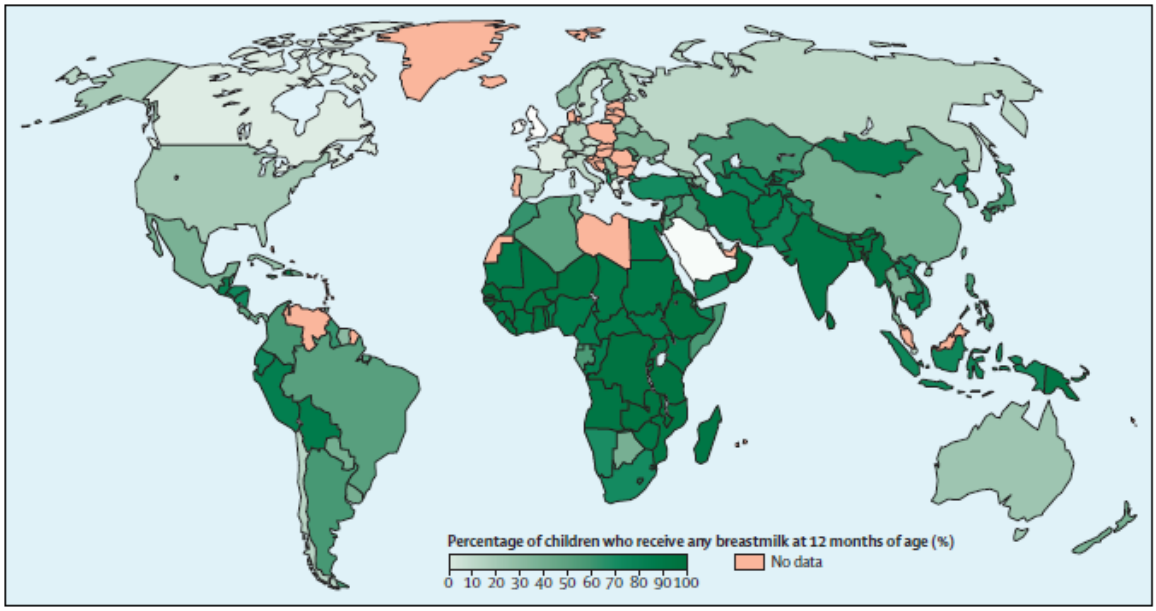


Figure 1. Distribution mondiale des nourrissons jusqu'à 12 mois de vie recevant l'allaitement maternel. Les données proviennent de 153 pays, entre 1995 et 2013. Source : Victora et al. (2016).

Bien qu'en constante évolution, les formules infantiles commerciales ne peuvent pas fournir les nombreux composants bioactifs du lait maternel. Ainsi, le lait maternel exprimé devient le premier choix pour les nourrissons vulnérables n'ayant pas accès à l'allaitement maternel. Le lait maternel contient des molécules bioactives qui protègent contre l'infection, l'inflammation et qui contribuent à la maturation immunitaire et digestive en début de vie (Donovan, 2016 ; Andreas, Kampmann, & Le-Doare, 2015 ; Labbok, Clark, & Goldman, 2004 ; Victora et al., 2016). Au cours des dernières décennies, les avantages du lait maternel comparé aux formules infantiles ont été démontré, ce qui encourage la mise en place de lactariums en particulier pour fournir du lait maternel de donneuses aux nourrissons vulnérables (Arslanoglu et al., 2013).

Aujourd'hui, il est bien connu que les formules infantiles diffèrent du lait maternel en termes de composition biochimique et de structure, ce qui peut avoir des conséquences nutritionnelles et à plus long terme sur la santé (Bourlieu & Michalski, 2015 ; Quigley & McGuire, 2014 ; Schanler, Lau, Hurst & Smith, 2005). La digestion, qui est l'étape intermédiaire entre l'aliment et l'organisme, est une étape clé dans la détermination des voies métaboliques et des conséquences nutritionnelles. Cependant, on connaît très peu le devenir des différentes émulsions laitières dans le tractus gastro-intestinal des nouveau-nés. Certains auteurs ont rapporté des différences en fonction de l'âge

gestationnel, l'âge postnatal et le type d'alimentation, notamment concernant la susceptibilité à la lipolyse, à la protéolyse, la vidange gastrique et la production de sécrétion (Roman et al., 2007 ; Armand et al., 1996 ; Cavell, 1981 ; Friedrikzon & Hernell, 1977). Néanmoins, les contraintes éthiques et techniques limitent la réalisation d'essais cliniques et, en particulier, l'accès à la phase intestinale. Avec le progrès des modèles *in vitro* simulant la digestion au cours des dernières années, le nombre d'études visant à mieux comprendre le processus digestif chez les nourrissons a augmenté, bien que cela reste un objectif ambitieux.

Même si les avantages de l'allaitement ont souvent été extrapolés au lait maternel exprimé, le stockage et les traitements technologiques modifient la composition du lait maternel et peuvent compromettre sa qualité nutritionnelle (Peila et al., 2016 ; O'Connor, Ewaschuk, & Unger, 2015). Parmi les traitements technologiques généralement appliqués au lait maternel pour des raisons sanitaires, la pasteurisation de Holder est recommandée mondialement, en dépit d'effets adverses tels que la réduction ou l'inactivation des lipases endogènes, des immunoglobulines, de la lactoferrine et de certains micronutriments (Peila et al., 2016 ; O'Connor, Ewaschuk, & Unger, 2015). En termes d'impacts physiologiques et nutritionnels, la pasteurisation du lait maternel peut diminuer l'absorption des lipides et le gain de poids moyen chez les nouveau-nés (Andersson, Savman, Blackberg, & Hernell, 2007 ; Thomaz, Goncalves, & Martinez, 1999 ; Williamson, Finucane, Ellis, & Gamsu, 1978). L'homogénéisation pourrait contrer le potentiel effet secondaire de réduction de lipolyse (due à l'inactivation de la lipase endogène) en augmentant la surface spécifique disponible pour l'adsorption de l'enzyme et, par conséquent, augmentant la digestibilité des lipides. Cela pourrait améliorer la croissance des nourrissons nourris avec du lait maternel pasteurisé et homogénéisé (Thomaz et al., 1999 ; Rayol, Martinez, Jorge, Goncalves, & Desai, 1993). Néanmoins, l'impact de ces traitements sur le comportement en digestion du lait maternel n'a jamais été étudié. Dans ce contexte, il semble important de répondre à la question de recherche suivante:

Est-ce que les traitements technologiques du lait maternel impactent sa digestion chez le nouveau-né ?

L'objectif principal de cette thèse était de répondre à cette question en étudiant l'impact des traitements physiques (pasteurisation de Holder et homogénéisation par ultrasons) sur la digestion gastro-intestinale du lait maternel chez le nouveau-né. L'impact des traitements était étudié *in vitro* et *in vivo* chez le nouveau-né, en évaluant les cinétiques de lipolyse, protéolyse et la déstructuration digestive des émulsions.

Malgré la richesse et la complexité du lait maternel aussi bien en termes de bioactivité que de nutriments, nous nous sommes focalisés sur la digestion des deux macronutriments que sont les

lipides et les protéines. Ce choix s'explique par l'importance que jouent les lipides et protéines pour supporter la croissance du nouveau-né (les lipides par exemple apportent 40-50% des calories totales du lait maternel) et pour leur bioactivité (protéines et peptides qui en dérivent par exemple). Ce choix était aussi justifié par l'intérêt récent portée à la structure du lait maternel, dont les lipides et protéines sont les principaux supports, et les potentiels effets de pré-programmation nutritionnelle récemment mis en évidence (Oosting et al., 2014 ; Oosting et al., 2012).

Pour atteindre cet objectif (illustré en Figure 2), des expériences de digestion *in vitro* et *in vivo* de lait maternel cru, pasteurisé ou pasteurisé-homogénéisé ont été réalisées (notés respectivement RHM, PHM and PHHM).

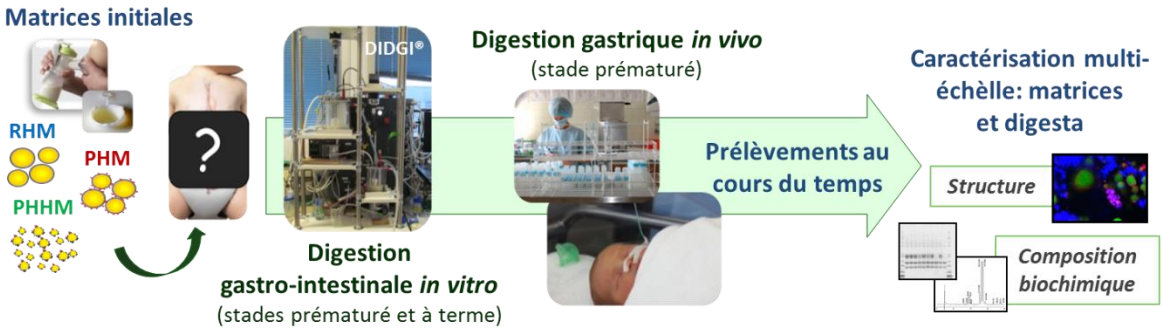


Figure 2. Stratégie pour investigation de l'impact des traitements pasteurisation de Holder et homogénéisation par ultrasons sur la digestion gastro-intestinale du lait maternel chez le nouveau-né. Laits maternels cru, pasteurisé et pasteurisé-homogénéisé sont notés respectivement RHM, PHM et PHHM.

En nous appuyant sur une revue récente et exhaustive sur les paramètres digestifs néonataux (Bourlieu et al., 2014), nous avons paramétré un digesteur dynamique afin qu'il reproduise aussi finement que possible les conditions gastriques et intestinales chez le nouveau-né à terme ou prématuré, à un mois de vie. Les données concernant l'étude *in vitro* sont présentées dans le chapitre 2. En outre, une étude *in vivo* a été conduite chez des nouveau-nés prématurés à l'Hôpital Universitaire de Rennes. Ces données sont présentées dans les chapitres 3 et 4. Cette étude *in vivo* nous a également permis de réévaluer des paramètres digestifs (à jeun ou en postprandial) chez le nouveau-né et de les comparer avec notre paramétrage initial du digesteur. Ces données sont détaillées en chapitre 5.

Le présent manuscrit est basé sur une série de six articles. A ce stade, quatre articles sont publiés dans des journaux à comité de lecture et les deux autres sont en préparation pour soumission. En outre, viennent compléter le manuscrit un premier chapitre présentant l'état de l'art autour de notre question de recherche, ainsi qu'un chapitre de discussion générale suivie d'une conclusion et des perspectives.

Cette thèse n'aurait pas été possible sans un réseau de collaborateurs que je tiens à mentionner dès le début de ce manuscrit, vu le rôle déterminant qu'ils ont joué dans la réussite de ce projet. Tout d'abord, cette thèse s'intègre dans le réseau européen du COST INFOGEST FA 1005, qui regroupait des chercheurs européens en science des aliments et nutrition cherchant à harmoniser les connaissances et méthodes sur la digestion des aliments. Ce projet a été mené en étroite collaboration avec l'Hôpital Universitaire de Rennes, ce qui a permis la mise en place de l'essai clinique ainsi que l'approvisionnement en lait maternel pour les digestions *in vitro*. Je tiens à saluer notamment l'implication forte de Patrick Pladys, Amandine Bellanger, Emelyne Dirson et leurs équipes. Des analyses des produits de lipolyse ont été conduites au laboratoire 'Enzymologie Interfaciale & Physiologie de la Lipolyse' (CNRS, Marseille) grâce à la collaboration avec Frédéric Carrière, qui a également fourni les enzymes digestives pour les digestions *in vitro*. Les analyses de lait maternel ont été réalisées dans l'UMR PhAN (INRA, Université of Nantes), grâce à la collaboration avec Clair-Yves Boquien. Une bourse de séjour scientifique a également été obtenue auprès du COST INFOGEST, ce qui a permis un échange d'un mois dans le Groupe Lipides, Bioactivité et Analyses des Aliments ('Grupo Lípidos Departamento de Bioactividad y Análisis de los Alimentos', CIAL, Madrid), expert en lipidomique et dirigé par Javier Fontecha.

Chapter 1 – Bibliographic review

1.1. Human milk: the ideal food in early nutrition

HM is a complex and dynamic biofluid recognized the gold standard in neonatal nutrition (Turck et al., 2013). The composition of HM highly varies throughout the lactation cycle, throughout the day, with maternal diet and length of gestation, and is believed to match with the infant requirements (Andreas et al., 2015; Gidrewicz & Fenton, 2014). **More than a source of nutrition, HM contains several bioactive factors which exert antimicrobial, immunomodulatory and digestive functions, compensating for immaturities in the neonatal immune and digestive systems.** These bioactive components contribute to the well-established short and long-term benefits of breastfeeding (Donovan, 2016; Andreas et al., 2015; Labbok et al., 2004; Victora et al., 2016). Particularly, breastfeeding is associated with a lower incidence of inflammatory bowel diseases and respiratory infections in the first weeks of life, and with a lower susceptibility to type II diabetes and obesity in adults (Victora et al., 2016; Turck et al., 2013).

Based on such evidence, the WHO recommends exclusive breastfeeding for the first six months of life, with continued breastfeeding after food diversification for up to two years or longer (WHO, 2001). HM from healthy women is able to support the growth and development of the exclusively-breastfed term infant and is the basis for determining infant nutritional requirements and dietary reference intakes (American Academy of Pediatrics, 2012). Thus, understanding the specific HM composition and its dynamics is crucial for optimal management of infant feeding at different gestational and postnatal ages, as well as for developing more biomimetic infant formulas.

1.1.1. Specific composition and structure organization

HM contains adequate nutritional, immunological and developmental components that assure optimal development of infants (American Academy of Pediatrics, 2012). **Table 1** shows the average composition of preterm and term HM at various postnatal ages. Compared to bovine milk, HM contains less protein, less ash and a higher level of lactose. Their protein and lipid fractions differ in terms of types, proportion and organization. For instance, in bovine milk the whey protein fraction is much lower than in HM (ratio casein/whey 80:20 and 30:70 *wt/wt*, respectively); in addition, the major whey protein is β-lactoglobulin, which is absent in HM (Guo, 2014c). With regards to the lipid fraction, its concentration, types of molecules (about 98% of triglycerides, TG) and organization under the form of milk fat globules (MFG) are similar in HM and bovine milk (Jensen, Hagerty, & Mcmahon, 1978). Nevertheless, their fatty acids (FA) composition is very different; notably, bovine

milk contains less long-chain polyunsaturated FA (LCPUFA) and more short-chain FA (SCFA) than HM (Guo, 2014c).

Table 1. Indicative average composition of human[a] and bovine[b] milk per 100 mL

Gross composition (%)	**Protein (g)**	**Fat (g)**	**Energy (kcal)**
Preterm human milk [c]			
1st wk	2.2	2.6	60
2nd wk	1.5	3.5	71
Wk 3-4	1.4	3.5	77
Wk 10-12	1.0	3.7	66
Term human milk [d]			
1st wk	1.8	2.2	60
2nd wk	1.3	3.0	67
Wk 3-4	1.2	3.3	66
Wk 10-12	0.9	3.4	68
Bovine milk	3.3	3.6	67

[a]Data of human milk obtained from a meta-analysis including 41 studies (Gidrewicz & Fenton, 2014)
[b]Data from (Malacarne, Martuzzi, Summer, & Mariani, 2002)
[c]26 studies and 843 mothers
[d]30 studies and 2299 mothers

Considering its structural organization, HM is a natural oil-in-water emulsion composed by native MFG dispersed in an aqueous phase containing a colloidal suspension of casein and other soluble components, such as serum proteins, carbohydrates and non-micellar mineral fraction.

The structure of HM casein micelles is not well known. Concerning their size, authors report a range from 30 – 70 nm, *i.e.* considerably smaller than those present in bovine milk (130 – 160 nm) (Chatterton, Nguyen, Bering, & Sangild, 2013; Kunz & Lonnerdal, 1989).

MFG presents a complex physicochemical structure, with polydispersed sizes ranging from 0.1 to 10 µm in mature HM (on average 4 µm) (Bourlieu & Michalski, 2015). TG are exclusively located in the core of MFG, which is surrounded by a trilayered biological membrane composed mainly by protein and phospholipid constituents, as a result of its secretion process (**Figure 3**). In the cytoplasm of the epithelial mammary cells (lactocytes), droplets of TG are surrounded by a coating consisting of a phospholipid/cholesterol monolayer with incorporated proteins. These lipid droplets are secreted from the cells by fusion with the apical plasma membrane, after being surrounded by secretory vesicles (Hernell, Timby, Domellof, & Lonnerdal, 2016). Thus, the primary membrane of MFG derives from the endoplasmic reticulum and the outer bilayer membrane derives from the apical plasma membrane of the lactocytes (Lopez, Cauty, & Guyomarc'h, 2015; Lopez & Ménard, 2011).

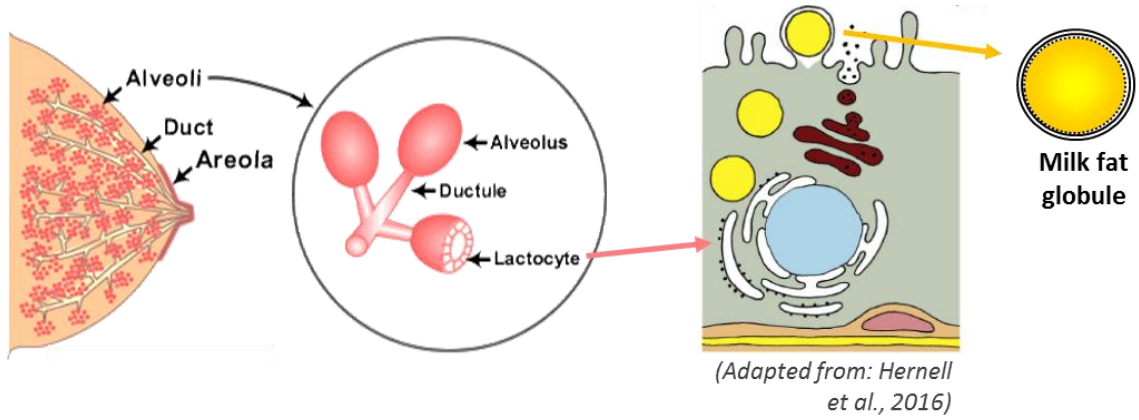


Figure 3. Secretion process of human milk fat globules

The MFG membrane is tensioactive and consists in potentially bioactive phospholipids, polar lipids, cholesterol, proteins, glycoproteins and enzymes, as shown in **Figure 4** (Bourlieu & Michalski, 2015; Lopez et al., 2015; Lopez & Ménard, 2011). In terms of physical-state, due to its heterogeneous chemical composition this membrane presents phase coexistence: lipid rafts (*i.e.* sphingolipids and cholesterol that aggregate along sections of the membrane) are in the lipid-ordered phase and are characterized by a high degree of rigidity; the lipid-disordered phase is composed by phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol) organized in a specific arrangement (Lopez & Ménard, 2011; Gallier, Gragson, Jimenez-Flores, & Everett, 2010).

1.1.2. Macronutrients

Lipids

The fat content in HM widely varies, about 30 – 50 g/L, contributing to 40 – 55% of its total energy and providing an adequate profile of essential FA (Michalski, 2013; Innis, 2007b). More than 98% of HM fat is present as TG, around 1% as phospholipids and 0.5% as sterol (mostly cholesterol). Some lipolysis products are also present in HM, *i.e.* diglycerides (DG), monoglycerides (MG) and free FA (Koletzko et al., 2001; Jensen et al., 1978).

HM typically presents complex FA profiles and a broad range of chain lengths, many of these being present in very low concentrations. In mature HM, around half are saturated FA (mainly palmitic acid), within SCFA and medium-chain FA (MCFA), *i.e.* C4:0 to C12:0, present in low concentrations (Michalski, 2013). HM is rich in long and very long-chain FA (LCFA and VLCFA, respectively), with oleic acid being the predominant monounsaturated FA and polyunsaturated FA (PUFA) including the essential FA linoleic (C18:2 ω-6, 6 – 22%) and α-linolenic (C18:3 ω-3, 0.1 – 4%). The ratio ω-6/ω-3 ranges between 6 and 15 (Lopez, Briard-Bion, Bourgaux, & Perez, 2013; Zou et al., 2012; Boue-Vaysse et al., 2009; Jensen, Ferris, & Lammi-Keefe, 1992). Among the factors affecting the FA profile (time

postpartum, gestational age, parity), diet is the most influent, thus leading to geographical variation in FA composition in links with local feeding practices (Zhang, 2014; Molto-Puigmarti, Castellote, Carbonell-Estrany, & Lopez-Sabater, 2011; Sala-Vila, Castellote, Rodriguez-Palmero, Campoy, & Lopez-Sabater, 2005; Jensen et al., 1992).

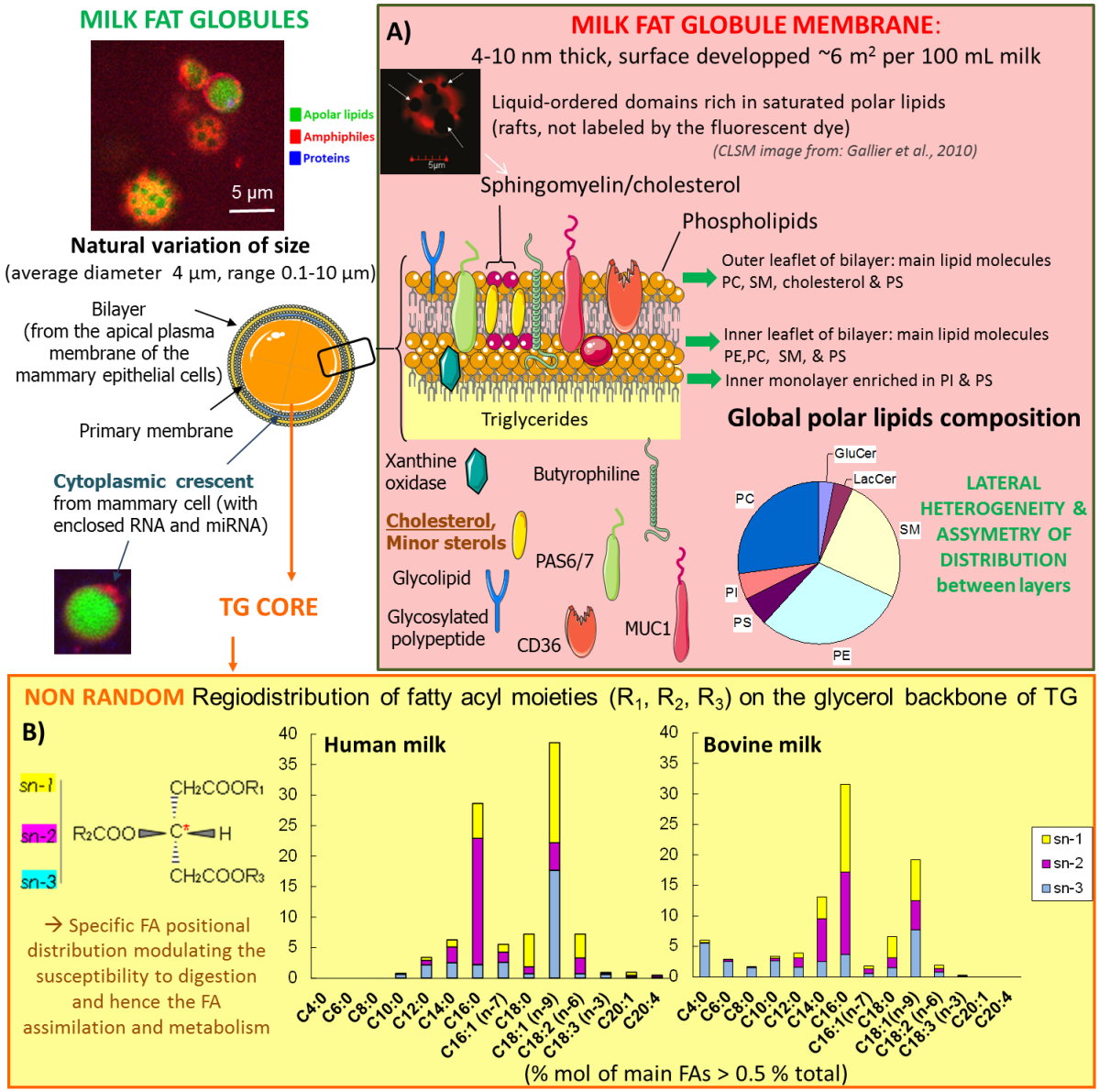


Figure 4. A) Organization of lipids in human milk: structure of the milk fat globule and its trilayered membrane. B) Comparative fatty acids composition and regiodistribution in human and bovine milk triglycerides. Adapted from Bourlieu & Michalski (2015). CD36, cluster of differentiation; CLSM: confocal scanning laser microscopy, FA, fatty acid; GluCer, glucosyl ceramide; LacCer, lactosyl ceramide; MUC1, mucin 1; PAS6/7, periodic acid Schiff protein (Lactadherin); PC, phosphatidylcholine, PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

Linoleic and α-linolenic acids are precursors of more complex LCPUFA, which have specific physiological functions and are crucial for normal development and growth. Notably, docosahexaenoic acid (DHA, C22:6 ω-3) is reported to play a role on retinal and brain development,

whereas arachidonic acid (ARA, C20:4 ω-6) is the precursor of prostaglandins and leukotrienes, critical regulators of metabolism (Innis, 2007a; Hamosh & Salem, 1998). Furthermore, DHA and ARA have been shown to have positive effects on the prevention of necrotizing enterocolitis (NEC) (Lu, Jilling, Li, & Caplan, 2007).

Polar lipids are major components of the MFG membrane, with a typical composition comprising glycerophospholipids and sphingolipids (**Figure 4**). Polar lipids also present a specific FA profile, including more unsaturated FA than TG; notably, LCPUFA such as DHA and linoleic acid are present (Michalski, 2013). Regarding sterol fraction, HM presents high cholesterol content (10 – 20 mg/100 mL) (Bourlieu et al., 2015a; Jensen, 1999), which is suggested to be linked with the protective role of breastfeeding against cardiovascular disease and notably lower blood cholesterol concentrations in later life (Michalski, 2013).

FA can be esterified on the three different positions of the glycerol backbone of a TG, which are defined as *sn*-1, *sn*-2 and *sn*-3 locations, and characterize the FA regiodistribution. It can be noted that this regiodistribution differs between bovine and HM (**Figure 4**B). This parameter affects the FA lipolysis and subsequent uptake (Zou et al., 2012; Bourlieu, Bouhallab, & Lopez, 2009), as digestive lipases can be regio- or stereo-selective. For instance, MCFA (C8:0 to C12:0) are mainly esterified in *sn*-3 position in HM, and thus selectively released by the *sn*-3 stereospecific human gastric lipase (HGL). These MCFA are then absorbed *via* the portal vein, transported to the liver to be β-oxidized, and thus constitute a rapid source of energy for the newborn (Bourlieu et al., 2015a). Oleic acid (C18:1 ω-9) is usually found at the *sn*-1 and *sn*-3 positions, which corresponds to the stereo-specificity of the human pancreatic lipase (HPL). Palmitic acid (C16:0) mostly present at the *sn*-2 position favors its intestinal absorption as *sn*-2 MG (Bourlieu & Michalski, 2015; Bernback, Blackberg, & Hernell, 1990; Jensen et al., 1978).

Proteins

HM is composed by a variety of proteins, providing a well-balanced source of amino acids (AA). Beyond their nutritional function, proteins have other utilities such as improving the digestion and uptake of other nutrients in HM, contributing to immune functions and to gut development, exerting antimicrobial activity, etc. Proteins are either solubilized in the soluble fraction (whey proteins, ~70 *wt*%), suspended in the colloidal phase (caseins, ~30 *wt*%), or bound to the MFG membrane (1 – 2 *wt*%) (Lonnerdal, 2016; Lonnerdal, 2003).

Table 2 shows protein fraction content in mature term HM.

Several recent revues detail the HM proteins composition, characteristics and function (Lonnerdal, 2016; Gidrewicz & Fenton, 2014; Chatterton et al., 2013; Lonnerdal, 2003). The present section will

focus on the major whey proteins in HM (α-lactalbumin, serum albumin, immunoglobulins, lactoferrin and lysozyme), as well as casein and AA fraction.

Table 2. Protein composition of term mature human milk[a]

Fraction	Term HM (g/L)
Whey proteins	5.3 – 6.6
α-lactalbumin	2.5
Lactoferrin	1.5
Immunoglobulins	1.2
Serum albumin[b]	0.4
Osteopontin	0.138
Lactoperoxidase	0.005
Caseins	3.5 – 4.4
β-casein	2.7
κ-casein	0.9
α_{s1}-casein	0.6
MFGM proteins	0.05 – 0.1

[a] Summarized from Chatterton et al. (2013)
[b] Data from Jensen (1995)
MFGM, milk fat globule membrane

Whey proteins

The high content of whey proteins in HM may have major relevance in infant nutrition, since several whey proteins have important physiological functions and are often able to bind vitamins, minerals and hormones (Chatterton, Rasmussen, Heegaard, Sorensen, & Petersen, 2004; Lonnerdal, 1989).

The **α-lactalbumin** is a calcium-binding protein but can also bind iron and zinc, and hence play a role on their absorption (Wada & Lonnerdal, 2014). Furthermore, it has been suggested to inhibit the growth of several pathogens (Lonnerdal, 2016). Despite high resistance to digestion due to calcium-binding stabilizing its structure (Chatterton et al., 2013), α-lactalbumin hydrolysis releases bioactive peptides (Wada & Lonnerdal, 2014), that have been shown to have antibacterial and immuno-stimulatory properties, as well as to encourage the growth of bifidobacteria (Lonnerdal, 2016).

Serum albumin has been suggested to be a passive carrier of ligands in the blood, *i.e.* FA, calcium, hormones and drugs (Lonnerdal, 1989). However, its physiologic function in HM remains unknown (Lonnerdal, 2016).

Immunoglobulins (*e.g.* IgA, IgG, and IgM) are known to exert antimicrobial activity compensating for the immaturity of the immune system in the newborn, protecting the infant from infections. IgA is the major immunoglobulin (~90%), and the more resistant to proteolysis (mainly the dimer sIgA).

This resistance is important for its functionality against several general pathogens in the gut, consisting a known process of acquiring immunity of the mother (Lonnerdal, 2016; Lonnerdal, 2003).

Lactoferrin is a ferric iron-binding glycoprotein present in high concentrations in HM, reported to resist the gastrointestinal digestion and thus to exert several bioactivities (Goldman, Garza, Schanler, & Goldblum, 1990). For these reasons, lactoferrin has been extensively studied over the last years (Donovan, 2016; Lonnerdal, 2013; Garcia-Montoya, Cendon, Arevalo-Gallegos, & Rascon-Cruz, 2012). Several activities have been attributed to lactoferrin, such as antimicrobial, immunomodulatory, anti-inflammatory, anticarcinogenic, enzymatic and gene regulating activities (Garcia-Montoya et al., 2012; Labbok et al., 2004). Both apo and holo-lactoferrin forms are absorbed in the human small intestine by an endocytic process, helped by a specific receptor (Jiang, Lopez, Kelleher, & Lonnerdal, 2011). Thus, lactoferrin is reported to play an important role in the iron absorption (Wada & Lonnerdal, 2014), even though the link between lactoferrin ingestion and iron status in infants remains unclear (Ke et al., 2015; King, Jr. et al., 2007; Hernell & Lonnerdal, 2002). Independently of iron saturation, the N-terminus of lactoferrin is able to bind other components, *i.e.* lysozyme, lipopolysaccharide, heparin, and DNA (Donovan, 2016). Despite of its well-known digestive resistance, lactoferrin can be partially hydrolyzed giving rise to an array of bioactive peptides. Notably, **lactoferricin** is reported to stimulate the immune system and to have antimicrobial activities (Garcia-Montoya et al., 2012; Labbok et al., 2004).

Lysozyme is an endogenous milk enzyme which has important protective functions in the infant, but does not participate on the HM digestion. Lysozyme is known to be a natural antibiotic having direct bacteriostatic and bactericidal action, either alone or in synergy with lactoferrin (Lonnerdal, 2003). Furthermore, lysozyme has been reported to have activities against HIV (Lee-Huang et al., 1999).

<u>Caseins</u>

The key caseins present in HM are κ- and β-caseins, while α_{s1}-casein subunit is present in low concentrations (unlike in bovine milk) (Lonnerdal, 2016). They are associated and linked together in the micelles with mineral ions (mainly calcium, phosphorus and magnesium), and are reported to exert protective functions, *i.e.* immuno-modulatory anti-inflammatory. Contrary to bovine milk, α_{s2}-casein is absent in HM (Chatterton et al., 2013).

The major casein, **β-casein**, derives several peptides that have been associated to antithrombotic, antihypertensive and opioid activities. Furthermore, during digestion, β-casein forms phosphorylated peptides able to bind divalent cations such as calcium and zinc, which is reported to facilitate their absorption. κ-casein is suggested to inhibit bacterial adhesion and to exert immunomodulatory activity (Lonnerdal, 2016).

Amino acids

Although several factors influence protein quality, **the AA composition of HM is recognized to be well balanced and able to support rapid growth of neonates** (Lonnerdal, 2016). Total AA are comprised of AA contributing to both protein nitrogen (protein-bound AA) and non-protein nitrogen (Zhang, Adelman, Rai, Boettcher, & Lonnerdal, 2013). Non-protein nitrogen content in HM (*e.g.* urea, uric acid, creatine, creatinine, nucleotides, free AA, small peptides) represents about 20 – 25% of total HM nitrogen, while in bovine milk it ranges from 3 – 5%.

Free AA represents 8 – 22% of the non-protein nitrogen fraction in HM, including mainly taurine, glutamic acid and glutamine, with the two later comprising nearly 50% of total free AA. One nutritional value of the non-protein nitrogen fraction is certainly that its free AA and small peptides are more rapidly bioavailable than in intact proteins (Atkinson, Schnurr, Donovan, & Lonnerdal, 1989). Indeed, the rapid absorption of free AA from HM contributes significantly to the initial changes in free AA levels in the infant plasma (Zhang et al., 2013).

Glutamic acid, for instance, is suggested to have many beneficial functions to the growing infant by providing ketoglutaric acid for the citric acid cycle, possibly acting as a neurotransmitter in the brain, and serving as a major energy substrate for intestinal cells (Zhang et al., 2013). Taurine is a non-protein sulfonic AA (Atkinson et al., 1989), one of the most abundant in the brain, retina, muscle tissue and organs throughout the body. Taurine has a variety of functions in the central nervous system, from development to cyto-protection (Ripps & Shen, 2012). In infants, taurine is considered essential for the normal development of the brain and retina, among other functions (Stapleton, Charles, Redmond, & Bouchier-Hayes, 1997); thus, IF is supplemented with taurine in order to reach quantities similar to that of HM.

Carbohydrates

Lactose, a disaccharide consisting of glucose covalently bound to galactose, is the main carbohydrate present in HM (around 70 g/L), and more generally the highest among all mammalian species. Beyond its source of energy, lactose seems to participate in the absorption of some minerals, notably by increasing the solubility of calcium salts in the intestine (Hendricks G.M. & Guo, 2014).

Oligosaccharides, classified among the non-nutritive bioactive factors in HM, are complex carbohydrates formed by three to ten monosaccharides (Andreas et al., 2015; Ballard & Morrow, 2013). Not digested by the infant, oligosaccharides have been largely studied for acting as prebiotics and playing an essential role in modulating the microbiota and preventing infections (Underwood et al., 2015; Newburg & Walker, 2007). Moreover, they may play a role on infant brain development (Bode, 2012) and calcium homeostasis (Lidestri, Agosti, Marini, & Boehm, 2003).

Other complex carbohydrates-based compounds are also present, such as glycolipids and glycoproteins.

1.1.3. Micronutrients

Despite important variations related to maternal diet and nutritional status, the micronutrient contents in HM from healthy mothers are generally considered as the normative standard for infant nutrition.

Fat-soluble vitamins (A, D, K and E) are involved in several essential functions, such as playing a role in bone metabolism and structure (D and K), contributing to immune system regulation (D), exerting antioxidant activity (E), etc. Water-soluble vitamins are more numerous: thiamin (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), folic acid (B9), cyanocobalamin (B12), ascorbic acid (C), biotin (B8), pantothenic acid (B5), inositol (B7), choline (Guo, 2014c). Almost all the fat- and water-soluble vitamins can be found in HM in adequate amounts to support normal infant growth, except vitamins D and K that are particularly low in HM and for which supplementation is necessary. Some authors also advice supplementation with other vitamins when the mother's diet is not optimal. For instance, infants breastfed by mothers on a strict vegetarian diet need supplementation of vitamin B12 (Guo, 2014c; Ballard & Morrow, 2013).

Minerals contribute to various physiological functions in the body, from providing structural components of body tissues to being essential parts in enzymes and other biologically important molecules. The macrominerals found in HM are sodium, potassium, chloride, calcium, magnesium, phosphorus and sulfate. The microminerals (or trace elements) are represented by iron, zinc, copper, manganese, iodine, fluoride, selenium, cobalt, nickel, aluminum, chromium and molybdenum (Guo, 2014c).

1.1.4. Endogenous enzymes and the pre-digestion of human milk

HM contains some enzymes favoring the digestion *versus* some anti-proteases favoring the resistance of some components to digestion. This paradoxical equilibrium is proposed as an evolutionary strategy (Goldman, 2000).

Bile salt-stimulated lipase (BSSL), also called as cholesterol ester hydrolase (CEH), is a lipase with wide substrate specificity and no regio-selectivity, able to hydrolyze all three ester-bonds of TG, DG, MG. In addition, BSSL is able to hydrolyze cholesterol esters and fat-soluble vitamin esters, such as retinyl esters (Hernell & Blackberg, 1994). BSSL requires the presence of primary bile salts to be active, and is also triggered by the presence of free FA (Bernback et al., 1990). Activity values from 20 to 35 U/mL were reported in HM at different stages of lactation (Hamosh, 1983). Besides enhancing

fat digestion during the digestive process, BSSL could contribute to fat pre-digestion since bile salts may be on sufficient concentration to trigger its action and initiate the lipolysis in HM (Forsyth, Donnet, & Ross, 1990). In fact, several authors have reported an increase in the lipolysis degree of HM during its storage (Bertino et al., 2013; Lavine & Clark, 1987; Bitman, Wood, Mehta, Hamosh, & Hamosh, 1983). **Although BSSL is more likely to act in the intestinal phase due to the presence of bile salts and more favorable pH, it could also act in the stomach triggered by bile salts carried by HM, and could be active in the wide range of pH (4.5 – 8), corresponding to the gastric pH in newborns** (Bakala N'Goma, Amara, Dridi, Jannin, & Carrière, 2012). This potential for contributing to fat digestion is believed to be important for newborn infants and particularly for preterm ones, who present limited coefficient of fat absorption due to their pancreatic and biliary system immaturity resulting in low production of pancreatic lipases and bile salts (Pamblanco, Ten, & Comin, 1987; Bourlieu et al., 2014). More details about the contribution of BSSL to the fat digestion and absorption will be discussed in section 1.4.3.

Lipoprotein lipase is another endogenous lipase identified in HM, with an activity around 0.4 to 1.8 U/mL of HM, *i.e.* about 100 times lower than BSSL (Freed, York, Hamosh, Mehta, & Hamosh, 1987; Freed, York, Hamosh, Sturman, & Hamosh, 1986; Hernell & Olivecrona, 1974). The function of lipoprotein lipase in HM or neonatal digestion is unknown.

Several **proteases and anti-proteases** have been identified in HM, forming a **complex proteolytic system** and taking part in the digestion of HM from the mammary gland to the digestive tract (Khaldi et al., 2014; Heegaard et al., 1997). The main proteolytic enzyme system in HM is plasmin, for which the activity is controlled by the levels of activators and inhibitors of its precursor plasminogen (Dallas, Underwood, Zivkovic, & German, 2012; Armaforte et al., 2010). For instance, type-1 plasminogen activator inhibitor and α1-antitrypsin are inhibitors that participate in the plasmin system by blocking the activator conversion of plasminogen to active plasmin (Dallas, Murray, & Gan, 2015). The protease inhibitors α_1-antitrypsin and anti-chymotrypsin are present in HM in relevant concentrations (Lindberg, Ohlsson, & Westrom, 1982), which can positively avoid or delay the breakdown of bioactive proteins either in undigested HM and during digestion (Lonnerdal, 2003).

There is no identified function of endogenous **α-amylase** in HM but some authors speculate that it is involved in the digestion of carbohydrates when complementary food is added to the diet (Lonnerdal, 2003). This activity could compensate for the low pancreatic amylase activity reported in the duodenal fluid of infants aged less than six months (Bourlieu et al., 2014).

1.1.5. Bioactivities beyond individual compounds

Nowadays it is recognized that the functional activities linked to HM may extend beyond the roles of its individual components. For instance, on the last years two constituents of HM have been studied for the importance of their components and complex structure organization: extracellular vesicles and MFG membrane.

Extracellular vesicles

Extracellular vesicles are spherical structures limited by a lipid bilayer, containing hydrophilic soluble components and secreted by many different cell types. Classified depending on their intracellular site of origin, endosomal and plasma membrane vesicles are named exosomes (50 – 100 nm) and microvesicles (100 – 1000 nm), respectively. They are recognized as potent vehicles for intercellular communication by transferring genetic material and messages encoded in proteins, lipids and RNA (Thery, Ostrowski, & Segura, 2009).

Recently identified in colostrum and mature HM, exosomes (~50 nm in diameter) were reported to contribute, *in vitro*, to immune regulatory functions (Admyre et al., 2007). Indeed, immune-related miRNAs, which are primarily synthesized in the mammary epithelium (Alsaweed, Lai, Hartmann, Geddes, & Kakulas, 2016), are enriched in HM exosomes and are resistant to general harsh conditions such as prolonged room temperature incubation, multiple freeze-thaw, RNase digestion and heat treatment (boiling) (Zhou et al., 2012). Furthermore, it has been demonstrated that HM exosomes resisted to *in vitro* gastrointestinal digestion and were further internalized by enterocytes, potentially affecting gene expression on their nucleus (Lonnerdal, Du, Liao, & Li, 2015).

Thus, although the role of the exosomes in HM *in vivo* remains to be elucidated, they are speculated to be transferable genetic material from mothers' milk to the infant (Zhou et al., 2012), and they are strongly suggested to positively influence the immune system of the infant (Bourlieu & Michalski, 2015; Zhou et al., 2012; Admyre et al., 2007).

A new kind of nanometer-sized lipid-protein assembly (~50 nm) named lactosome has also been identified in HM (Argov-Argaman et al., 2010). Also made up of membrane vesicles, it is still unknown if these lactosomes are exosomes. They have a density equivalent to plasma high-density lipoproteins, present a different protein and lipid profile compared to MFG, are devoid of a TG core and are enriched in immuno-modulatory proteins. Although their biological role remains unclear, taking into consideration their composition and drawing parallel with exosomes, they have been suggested to play a role in the infant's metabolic and immune health (Bourlieu & Michalski, 2015; Gallier et al., 2015a; Argov-Argaman et al., 2010).

Human milk fat globule membrane

The composition and organization of MFG membrane have been largely studied due to its nutritional, physiological and health benefits. A literature survey in the Web of Science displays 781 publications when researching the key words 'milk fat globule membrane' over the last 20 years, with a notable increase in their number over the last 5 years. When refining the research towards the key words 'human' and 'milk fat globule membrane', 204 articles were found. Several comprehensive reviews are available on this topic (Hernell et al., 2016; Bourlieu & Michalski, 2015; Lopez et al., 2015).

Individual compounds of the MFG membrane often studied comprise choline, gangliosides, cholesterol, sialic acid. Polar lipids, the major components of the human MFG membrane, impact cell metabolism and present several bioactivities such as brain development and cognitive functions, immunity and gut health (Lonnerdal, 2014; Kullenberg, Taylor, Schneider, & Massing, 2012; Hirabayashi & Furuya, 2008).

Remarkably, recent advance in the characterization of the membrane proteome, with special emphasis on glycosylated proteins, allowed the identification of more than 190 intracellular, extracellular and membrane associated proteins in the human MFG. These proteins are mainly involved in lipid and energy metabolisms, cell communication and signal transduction, and immune function (Liao, Alvarado, Phinney, & Lonnerdal, 2011). Glycoproteins have been reported to prevent infections in breastfed infants. Indeed, Peterson et al. (1998) demonstrated that two MFG glycoproteins implicated in prevention of infection, MUC-1 mucin and lactadherin (PAS6/7) maintained their integrity in the stomach of HM-fed preterm infants.

Some benefic effects of the MFG membrane have been associated specifically to the combination of its components and their structure organization, such as digestive resistance of some components and protection against oxidative stress (Bourlieu & Michalski, 2015). Indeed, MFG membrane configuration is reported to protect some bioactive molecules and retard their digestive breakdown, allowing them to exert their function (Zhang et al., 2014). For instance, the limited susceptibility of sphingomyelin to digestion favors the persistence of sphingomyelin–cholesterol complexes in the proximal part of the intestine. In addition, sphingomyelin and its metabolites (ceramide, sphingosine, ceramide-1-P and sphingosine-1-P) influence TG hydrolysis, cholesterol absorption, lipoprotein formation and mucosal growth in the gut (Bourlieu et al., 2015a). Still, the protective structure of the MFG membrane contributes to the high oxidative stability of expressed HM, in despite of its high content in LCPUFAs (Michalski et al., 2013).

In piglets, IF with milk lipids stabilized by MFG membrane extracts positively modified the microbiota composition and the mucosal immunity, as compared to formula with a blend of vegetable lipids (Le

Hueron-Luron et al., 2016). In infants and children, clinical studies have investigated the safety and efficacy of IF supplementation with MFG membrane (Hernell et al., 2016). Results are mostly positive and demonstrate benefits in terms of cognition, behavior, gut health and immunity (Timby et al., 2015; Timby, Domellof, Hernell, Lonnerdal, & Domellof, 2014; Timby, Lonnerdal, Hernell, & Domellof, 2014; Veereman-Wauters et al., 2012; Zavaleta et al., 2011). Although the interventions strategy and MFG membrane concentrates added are still too heterogeneous, these results are supported by available evidence about the beneficial effects of individual components of MFG membrane. Thus, adding native MFG membrane to IF is a promising step towards more HM biomimetic IF.

1.1.6. Human milk as a dynamic biologic fluid

HM composition is complex and influenced by several factors, such as gestational and postnatal ages, maternal diet and others (Andreas et al., 2015; Ballard & Morrow, 2013).

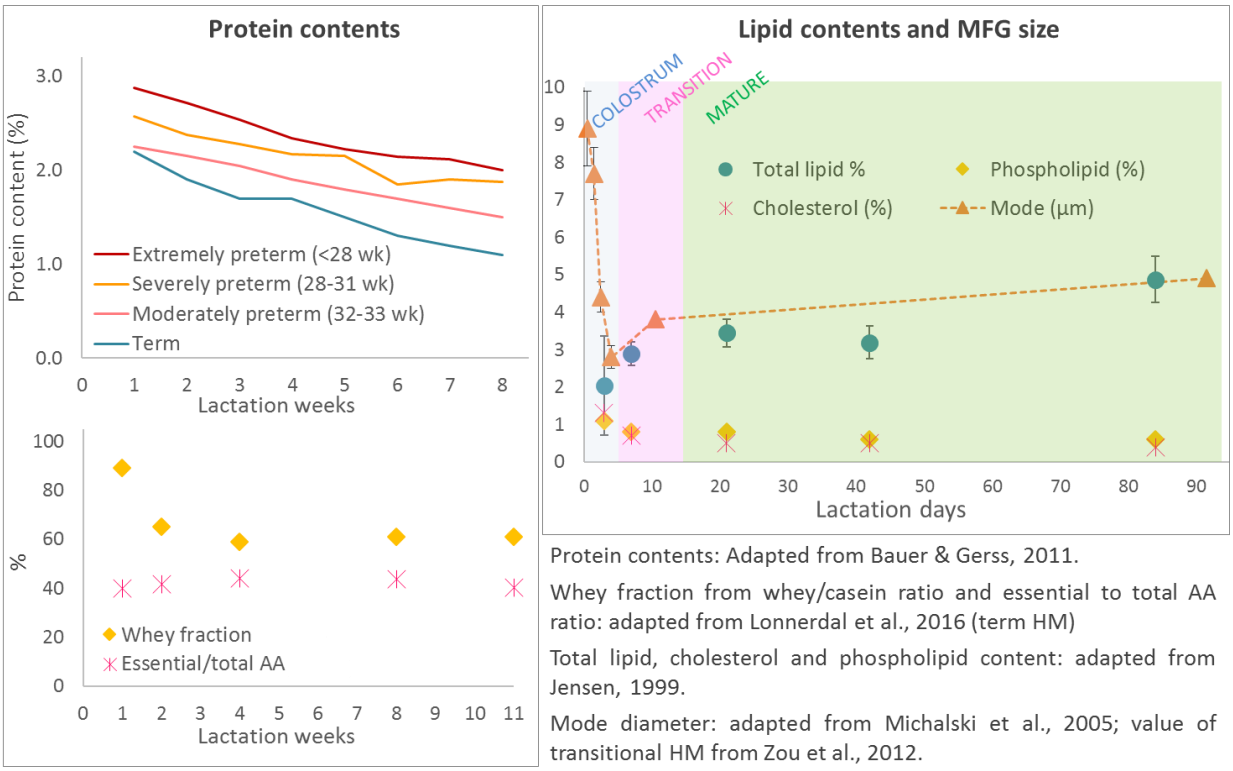
Gestational and postnatal ages

Recently, a meta-analysis providing data on HM composition from 26 preterm studies (843 mothers) and 30 term studies (2299 mothers) confirmed that gestational (preterm *versus* term) and postnatal ages were found to be important predictors of HM content (Gidrewicz & Fenton, 2014). Part of the data from this study was summarized in **Table 1**, section 1.1.1.

Overall, at the same postpartum period, preterm HM tend to be higher in protein and fat than term HM (Bauer & Gerss, 2011; Anderson, Atkinson, & Bryan, 1981). However, after 10 to 12 weeks of lactation this difference tends to disappear (decrease of protein and a trend of increase in fat content over the lactation) (Gidrewicz & Fenton, 2014). These changes suggest that maternal lactogenic compensatory mechanisms take place, adapting the HM composition to the infant needs (Goldman, 2000).

According to the postnatal age, HM can be categorized into three types: colostrum, transitional and mature (Bitman et al., 1986). After delivery, the first fluid produced by mothers is named colostrum (1 to 5 days). Afterwards, transitional HM is typically produced from six days to two weeks postpartum, and mature HM from the third week on. They differ in terms of composition and structure (**Figure 5**). Compared to transitional and mature HM, colostrum is very distinct in appearance and produced in low quantities. It presents lower concentrations of lactose and is especially rich in immunologic components (*e.g.* secretory IgA, lactoferrin, leucocytes) and developmental factors (*e.g.* growth factors), which suggest immunologic functions supporting intestinal development rather than a purely nutritional function (Castellote et al., 2011; Pons, Bargallo, Folgoso, & Sabater, 2000). The transitional HM has a composition closest to mature HM, which keep changing over the lactation but less dramatically.

Independently of the gestational age, protein levels decrease over the first 4 to 6 weeks of lactation, at least (Lonnerdal, Erdmann, Thakkar, Sauser, & Destaillats, 2016; Bauer & Gerss, 2011). Remarkably, the whey protein fraction is high during the early stages of lactation (about 90%) and decreases with time (Lonnerdal et al., 2016). Thus, the general ratio of 60:40 *wt/wt%* of whey proteins to caseins reflects the HM composition after one month of lactation. Because the AA contents of whey proteins and casein differ, HM AA content also changes as infants mature but the essential to total AA ratio remains quite stable (Lonnerdal et al., 2016; Zhang et al., 2013).



Protein contents: Adapted from Bauer & Gerss, 2011.

Whey fraction from whey/casein ratio and essential to total AA ratio: adapted from Lonnerdal et al., 2016 (term HM)

Total lipid, cholesterol and phospholipid content: adapted from Jensen, 1999.

Mode diameter: adapted from Michalski et al., 2005; value of transitional HM from Zou et al., 2012.

Figure 5. Protein and lipid changes over lactation.

Fat is the most highly variable macronutrient of HM, in terms of concentration. Contrary to proteins, lipid content increases over the lactation (Bourlieu et al., 2015a; Jensen, 1999). In terms of FA profile, MCFA (C8:0 to C14:0) and saturated FA were reported to increase between the colostrum and the two other lactation periods, while monounsaturated FA decrease (Molto-Puigmarti et al., 2011; Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). LCPUFA do not vary significantly over the lactation (Lopez-Lopez et al., 2002) but is reported to be higher in preterm than in term HM, including DHA (Bokor, Koletzko, & Decsi, 2007). The specific regiodistribution of FA in HM TG does not evolve with lactation stage (Martin, Bougnoux, Antoine, Lanson, & Couet, 1993).

As a result of the increase of total lipid, membrane of MFG becomes a limiting factor and thus the MFG size increases. Michalski et al. (2005) reported a higher proportion of large MFG in early colostrum, with a mode (most frequent size in the distribution in volume) rapidly decreasing from 8.9 ± 1.0 µm at 12 h post-partum to 2.8 ± 0.3 µm at 96 h post-partum. The mode then increased regularly but slowly in mature milk, from values around 5 µm at 3 months lactation up to 6.5 µm at 18 months. In mature HM, the larger MFG leads to a small membrane surface/TG content ratio. Thus, the reduction of the MFG membrane fraction over lactation results in a reduction of phospholipids/TG and cholesterol/TG ratio (Bourlieu et al., 2015a; Jensen, 1999; Harzer, Haug, Dieterich, & Gentner, 1983).

Maternal diet

Nutrients of HM can be synthesized by the lactocytes in the breast or actively transported from the blood to the milk (McManaman & Neville, 2003). The macronutrient composition of HM is remarkably stable across different ethnicities, except for fat content. This macronutrient varies the most, both among mothers and ethnicities (Jenness, 1979), and very likely due to differences in maternal diet (Lopez-Lopez et al., 2002).

The lipids vary with the maternal diet in terms of overall content and/or in terms of FA profile (Delplanque, Gibson, Koletzko, Lapillonne, & Strandvik, 2015; Zhang, 2014). The synthesis of SCFA and MCFA would be enhanced by high-carbohydrates and low-fat diets (Michalski, 2013), while PUFA come from reserves or maternal diet (Zhang, 2014). The high variability on the level of LCPUFA in HM is due to the dietary content undertaken by mothers. In the last decades, an increase in the maternal intake of ω-6 (*e.g.* α-linolenic FA) has resulted in marked increase of the ratio ω-6/ω-3 (Delplanque et al., 2015; Zhang, 2014). Since this ratio is also reported to increase over the lactation (Molto-Puigmarti, Castellote, & Lopez-Sabater, 2011), it could overtake the recommended range of 5:1–15:1 established by the ESPGHAN Committee on Nutrition. The sub-optimal intake of ω-3 FA in North American populations, for example, is considered as a reason for DHA supplementation for breastfeeding women (Ballard & Morrow, 2013).

Micronutrients in HM can vary depending on maternal diet and stores, and are frequently categorized into two groups. The first one comprises those with most interest in nutrition, being affected by maternal depletion or supplementation. They are thiamin, riboflavin, pyridoxine, cyanocobalamin, choline, retinol, vitamin A, vitamin D, selenium and iodine. The second group is little affected by maternal diet and comprises folate, calcium, iron, copper and zinc. The mother becomes depleted when intake is not sufficient to cover the amount secreted in HM and may need supplementation (Allen, 2012).

Other factors

HM composition even varies during a single meal: the energy content of HM is lower at the beginning of a feeding, called foremilk, than at the end, hind milk. Indeed, fat content progressively increases during the feeding process, whereas lactose decreases (Andreas et al., 2015). Other factors such as age of mother, weight gain during pregnancy and parity have been suggested to influence HM composition, but no large impacts were identified (Andreas et al., 2015).

1.2. How optimally nourish infants without access to breastfeeding?

The recommendation of exclusive breastfeeding is reinforced during the first month (neonate or newborn infants, < 28 days of postnatal age) not only for preterm infants (born before 37 weeks of gestation), but also for term infants (born after 37 and before 42 weeks of gestation). However, cultural, economic, medical or environmental factors can make breastfeeding inaccessible or limited (Guo, 2014a). The substitute chosen may respect the physiologic constraints of the infant, meeting his nutrient requirements and respecting his digestive immaturity. Expressed HM, if available, is the best option (Arslanoglu et al., 2013). This practice is increasing, either at home or in the hospital, where high-risk infants can receive HM from the own mother or pasteurized HM from anonymous donors.

If expressed HM is not available, most of the infants without access to breastfeeding are fed infant formula, that are developed to be as close as possible to HM in terms of composition (Guo, 2014b). These topics will be discussed below in this section.

1.2.1. First months feeding and health outcomes

The **feeding type in the early life** modulates not only short-term outcomes such as growth and development, but also long-term health outcomes along life. Factors potentially influenced by the feeding type during this period include neurodevelopment, metabolic health, immune competency, atopic disease, establishment of the mucosal microbiome and behavioral responses to foods and eating (Raiten et al., 2016; Arrieta, Stiemsma, Amenyogbe, Brown, & Finlay, 2014; Maslowski & Mackay, 2011).

Although evidence regarding the risk/benefits of dietary alternatives to breastfeeding is considered insufficient (Raiten et al., 2016), **pasteurized HM from HMBs is recognized to be the best option for vulnerable hospitalized neonates who need enteral nutrition.** Administration of pasteurized HM has

been shown to reduce mortality and improve development and nutrition when compared to IF (Quigley & McGuire, 2014; Boyd, Quigley, & Brocklehurst, 2007; Wight, 2001). Compared to HM, IF feeding increases the risks of NEC, atopy, diabetes mellitus, childhood obesity and reduces the cognitive development (Quigley & McGuire, 2014; Guo, 2014a).

NEC is a significant cause of morbidity and mortality in preterm infants, and thus one of the main preoccupations regarding short-term outcomes. In addition, the high risk of neurological abnormalities and negative neurodevelopmental outcomes presented by preterm infants is often linked to the type of nutrition (Arslanoglu et al., 2013). Overall, this supports the use of pasteurized HM instead of IF, when breastfeeding is not available. In particular, a meta-analysis from Boyd et al. (2007) found a lower risk of NEC but slower growth in the early postnatal period for infants fed banked-HM compared to IF. Rozé et al. (2012) found that infants fed both mothers' and banked-HM compared to infants fed IF presented slower initial growth, but further better neurologic outcomes; these findings are known as the **"breastfeeding paradox"**.

Recently, HM components and structure have been explored regarding possible epigenetic mechanisms explaining their benefits for infants (Verduci et al., 2014). However, there are not enough studies to clarify the link between HM and gene expression.

As an example of **pre-programming concept,** a study in mice recently demonstrated that the exposure in early life to a concept infant formula biomimetic of native MFG reduced the obesity risk in adult life (Oosting et al., 2014; Oosting et al., 2012).

It is now clear that the microbiome in early in life may regulate host physiology, influencing risk for a variety of pathophysiological effects into childhood and even adult life. Immediately after birth, the infant acquires an intestinal microbiota that is shaped by both genetics and environmental settings, including a significant role of maternal and early postnatal diet (Bourlieu et al., 2015a; Groer et al., 2014). Compared to IF, breastfeeding drives the infant intestinal microbiota toward a more beneficial community composition dominated by *Bifidobacterium* and *Lactobacillus* (Bergstrom et al., 2014; Groer et al., 2014).

1.2.2. Specificities of the preterm infant

Preterm infants are more vulnerable than term ones due the high susceptibility to infections, digestive immaturity, complications due to impaired respiration, poor body temperature regulation, among other factors (WHO, 2015). Preterm infants and especially preterm newborns face specific risks and need special attention to deal with their temperature instability, feeding difficulties, low blood sugar, infections, breathing difficulties, etc. (WHO, 2012). Preterm birth is a direct risk and leading cause of newborn mortality, besides increased risk for long-term physical, neuro-

developmental and behavioral outcomes (Blencowe et al., 2013). This is even more true for preterm infants born before 32 weeks of gestation, also classified based on their low birth weight (< 2.5 kg) or very low birth weight (VLBW, < 1.5 kg), usually presenting higher mortality risk and needing more careful nutritional management (WHO, 2011). HM feeding is particularly beneficial to these vulnerable infants as HM-fed preterm present lower mortality rates, shorter hospital stay, lower incidence of infections, better neurodevelopment and better health in adulthood (Moro et al., 2015; American Academy of Pediatrics, 2012; Schanler et al., 2005; Wight, 2001).

Preterm newborns often need tube feeding and cannot be breastfed. In these cases, the mother's fresh expressed milk becomes the first choice, as proposed by Arnold (2006) (**Figure 6**). His diagram illustrates the hierarchy of the feeding choices for low birth weight infants based on the WHO recommendations. Infant formulas are the last options.

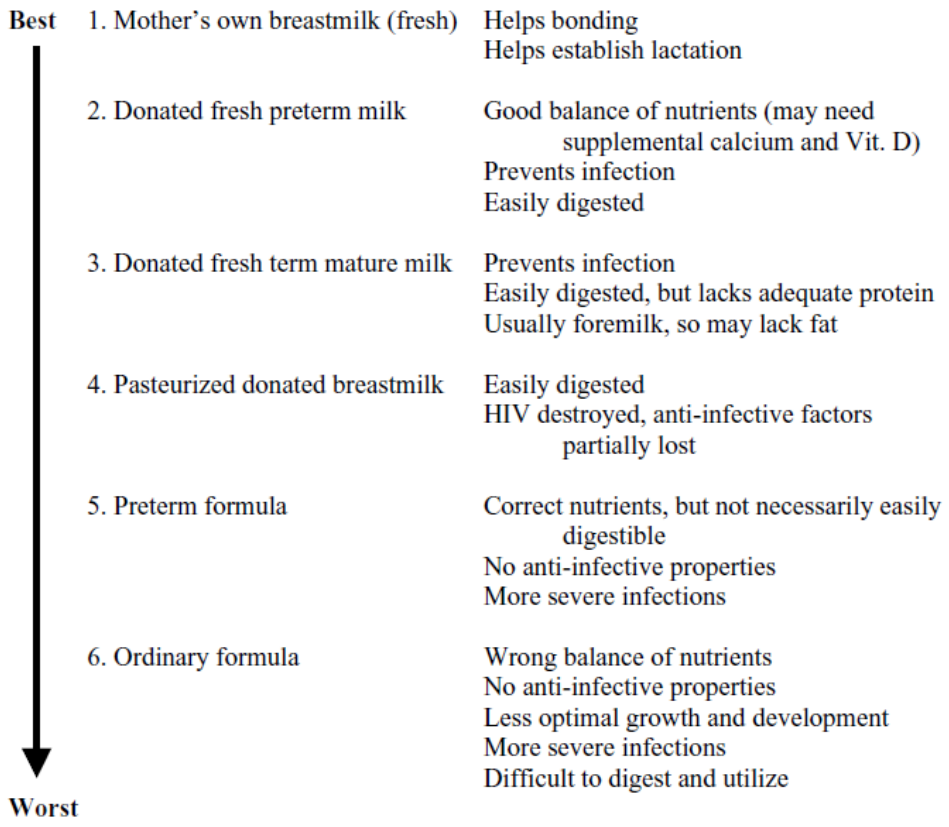


Figure 6. Hierarchy of the feeding choices for low birth weight infants. Source: Arnold (2006).

1.2.3. Human milk fortification

Fortification of HM (addition of macro and micronutrients) is conducted to achieve the special nutritional requirements of the preterm infant and is considered as a strategy to assure adequate postnatal growth rate (Rochow, Landau-Crangle, & Fusch, 2015; Agostoni et al., 2010; Arslanoglu, Ziegler, & Moro, 2010). However, this practice is often debated and there is no consensus about the

fortification method (Raiten et al., 2016; Moro et al., 2015; Rochow et al., 2015). Indeed, the use of bovine milk–based fortifiers potentially increases the risk of feeding intolerance and NEC (Adamkin & Radmacher, 2014). In addition, some studies highlight the risk of adverse effects such as metabolic acidosis (Rochow et al., 2011) and increase of osmolarity with consequent gastrointestinal intolerances (Kreissl et al., 2013; Pearson, Johnson, & Leaf, 2013).

Some authors support that to be effective and avoid adverse effects, the fortification should be targeted in order to adjust the macronutrient contents to the infant needs after HM analyses (Morlacchi et al., 2016; Arslanoglu, Moro, Ziegler, & The Wapm Working Group On Nutrition, 2010). Compared to standard fortification (addition of fixed doses of fortifier without taking into account the caloric and nutrient content of the HM), the individualized targeted fortification is reported to limit the risk of macronutrient under-intake without increasing the risk of adverse effects, and appears to promote growth in VLBW infants during the hospital stay (Morlacchi et al., 2016).

Milk analyzers using mid-infrared or near-infrared spectroscopy are commercially available, allowing the measurement of fat, protein, lactose and the resulting estimation of calories from small aliquots (1 – 1.5 mL). Importantly, before being introduced in the clinical routine of HMB, each apparatus needs calibration and validation on HM against standard methods (Billard et al., 2015; Fusch et al., 2014). Even though infrared analysis seems to be a promising tool for fat and protein measurements, lactose measurement still needs major adjustments, which subsequently impairs calorie estimation (Rochow et al., 2015; Fusch et al., 2014). Some authors speculated that oligosaccharides, abundant in HM, could affect the lactose measurements (Smilowitz, Gho, Mirmiran, German, & Underwood, 2014). Overall, the accurate nutritional quality control of HM remains difficult to implement in the clinical routine.

1.2.4. Human milk banks

According to the American Medical Association (2001), HMB is a ‘specialized center, obligatorily linked to a maternal and/ or children’s hospital, responsible for promoting breastfeeding and execution of collection, processing and quality control of HM for distribution under prescription from the doctor or dietician. Thus, they recruit and screen HM donors, collect, store, treat and distribute the HM, allowing HM adequate supply to vulnerable hospitalized infants that cannot be breastfed (Picaud, 2015).

The establishment of HMBs is extensively encouraged by public health policies and officially recommended (Arslanoglu et al., 2013; American Academy of Pediatrics, 2012; WHO & UNICEF, 2003). Notably, the presence of a HMB in hospital increases the breastfeeding rates at discharge and decreases the IF use during the first weeks of life (Arslanoglu et al., 2013). Thus, HMBs and related

associations are present in numerous countries, with well-established regulation and specific guidelines for every procedure, which assure safety and high quality of banked-HM. The regulations relate to all the steps of handling, processing and storing of HM, differing depending on locations, organization and resources (HMBANA, 2015; Guo, 2014a; Arslanoglu et al., 2010; Grovslien & Gronn, 2009; Hartmann, Pang, Keil, Hartmann, & Simmer, 2007; Arnold, 2006).

Donors and receipts of banked-human milk

The banked-HM is provided by the infant's mother (direct donation) or by anonymous donors (anonymous donation). The direct donation occurs mostly when the hospitalized infant did not yet develop the suck-swallow-respiration coordination and then need enteral nutrition through a nasogastric tube (Lau, 2015; Rayyan, Rommel, & Allegaert, 2015). In these cases, mothers usually collect their breast milk, which will be either refrigerated if administrated within 24 up to 72 h (depending on the local practices) or frozen (-20 °C) and sent to the HMB for storage and processing. Anonymous donors give their extra-HM after careful screening for general health, alcohol, medication or drug consumption and infections (*i.e.* HIV, hepatitis, syphilis, etc.) (Picaud, 2015; Hartmann et al., 2007). Thus, receipts of banked-HM are vulnerable hospitalized infants, mainly preterm and VLBW neonates that cannot be breastfed (Picaud, 2015). Other potential receipts are vulnerable infants with different problems, such as severe allergies, feeding intolerance, gastrointestinal problems, etc. (Wight, 2001).

A constraint in the use of pasteurized HM from anonymous donor for preterm infants is a discrepancy between their needs and donor's lactation stage, most often mothers of term infants beyond one month postpartum (Gidrewicz & Fenton, 2014; Ballard & Morrow, 2013). As discussed in the session 1.1, HM nutritional content is changing with the gestational age and stage of lactation in order to meet the infants' needs. In term HM, the levels of protein content, for instance, is lower compared to preterm HM, which thus leads to fortification prescription.

Holder Pasteurization: recommended by worldwide guidelines

Although HMB practices can vary depending on the country, the most common practice to assure the microbiological quality of the banked-HM is the Holder pasteurization. It is a long-time and low-temperature method, it consists in heating pooled bottles of HM in a water bath at 62.5 °C during 30 min, followed by rapid cooling (HMBANA, 2015; Picaud, 2015; Guo, 2014a). However, the time taken to reach this temperature and to cool the milk is not standardized as it varies depending on several factors, such as material of bottle and milk volume. Without any contact with the milk, this physical and thermal process is able to destroy infectious agents such as immunodeficiency virus, human T-

lymphoma virus, cytomegalovirus, tuberculosis and other bacterial contaminants such as *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* (HMBANA, 2015).

There is a long process from the HM expression by mothers, handling and processing by the HMB to delivery to infants (**Figure 7**). Some steps of this process, *i.e.* screening and serology of mothers, depend on the type of donation (anonymous or direct). The local practices also can highly variate, *i.e.* pool of independent milk expressions from the same donor or pool of HM from multiple donors.

Briefly, after expression following instructions on hygienic methods, HM is stored as frozen in the HMB (-20 °C) for up to three months (ideal) to one year (acceptable), depending on HMB individual procedures. Thawing is usually carried in controlled room temperatures for a few hours or overnight at 4 °C. Then HM is pooled, bottled and Holder pasteurized. A sample is taken for bacteriological analyzes before and after pasteurization. The pasteurized HM is then frozen and cannot be delivered before conform results from bacteriological analyses are confirmed (Sousa, Delgadillo, & Saraiva, 2016). Thawing procedure is again carried before delivering milk to infants, which can last up to 24h. Noticeable, two cycles of freeze-thaw are applied during HM storage: first from milk expression to pasteurization and last from pasteurization to administration (**Figure 7**).

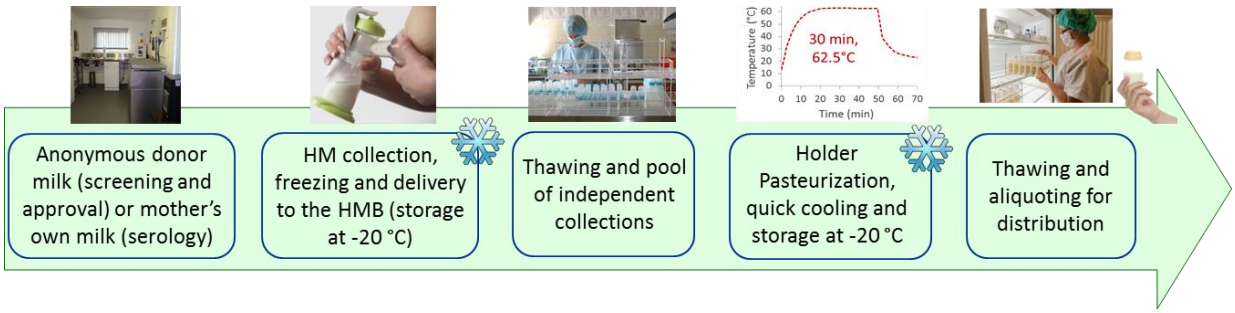


Figure 7. Banked-human milk: steps from collection to distribution

1.2.5. Infant formulas

Highlighting HM composition and structure specificities have provided a basic knowledge guiding new priorities for the improvement of IFs. IFs are manufactured milk-based formulas in powder or liquid form, mostly composed of defatted bovine milk, vegetable oils and other purified compounds such as bovine whey proteins, specific FA, AA and micronutrients. Despite the great advances in the optimization of IF composition, they cannot provide all the numerous immune protective and bioactive factors present in HM. Furthermore, their structure remains very different from that of HM, which leads to a difference in the efficiency of digestion and assimilation of nutrients (Bourlieu et al., 2015a; Bourlieu et al., 2015b; Armand et al., 1996).

The IF emulsion is based on several reassembled dairy and non-dairy fractions, resulting from successive technological operations (**Figure 8**). IF are composed of submicronic droplets (0.3 – 0.8

µm) stabilized by a neoformed membrane mainly based on milk proteins (**Figure 8**). Non-dairy tensioactives (mainly vegetable or functionalized lecithins or esters of partial glycerides) are usually added to stabilize the emulsion. The regiodistribution of FA on TG in IF differs largely from the one in HM, since the source of fat in IF is a combination of blended vegetable oils which aims at mimicking the FA composition in HM (Zou, Pande, & Akoh, 2016; Bourlieu et al., 2015a).

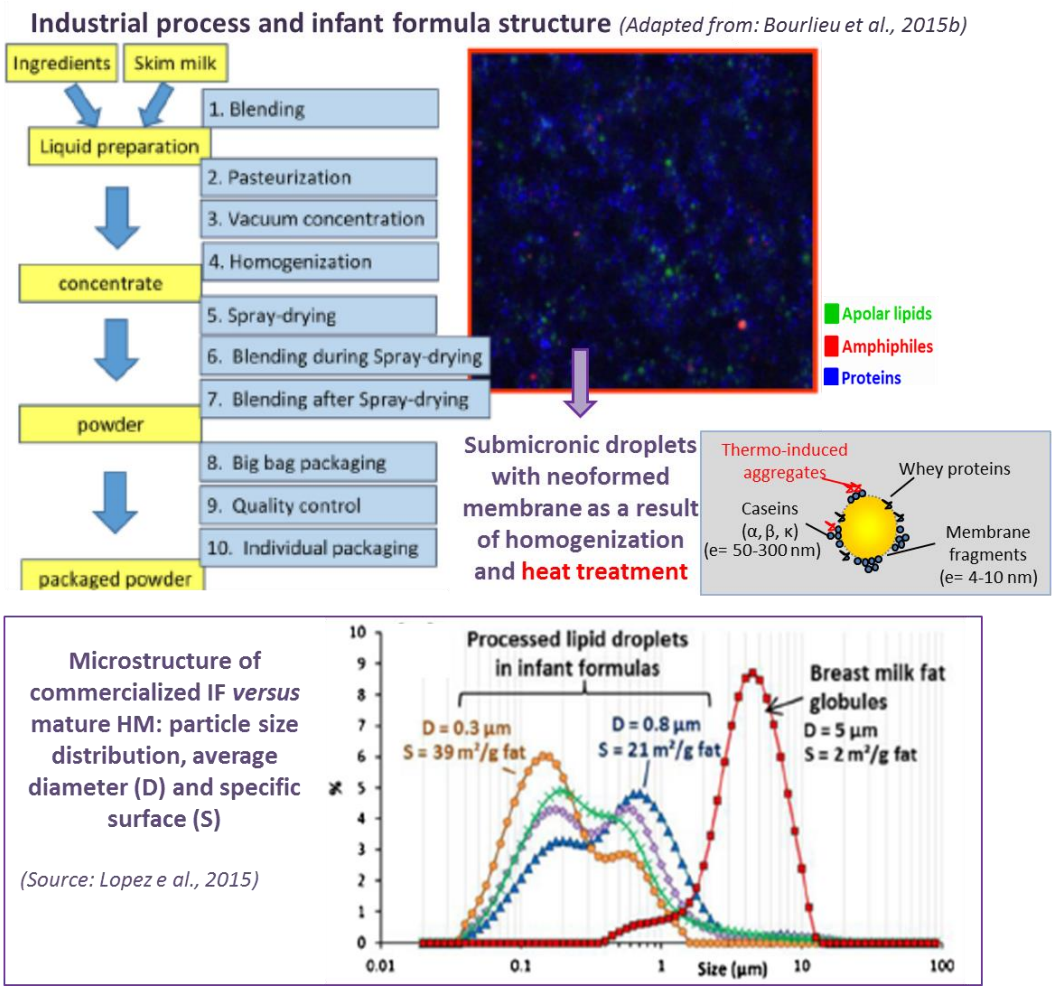


Figure 8. Industrial process for infant formula fabrication and impact on the emulsion structure.

Since HM is a complex and dynamic matrix very difficult to mimic, the addition of bioactive components in IF and their structure optimization have been considered by many authors and industries (Zou et al., 2016; Gallier et al., 2015a; Committee on the Evaluation of the Addition of Ingredients New to Infant Formula, 2004). Notably, the addition of LCPUFA, lactoferrin, nucleotides, essential AA, oligosaccharides, prebiotics, probiotics, MFG membrane fragments, modification of the TG structure of fats and oils to mimic HM TGs, as well as the development of specialized formulas for specific needs, have become popular (Mudd et al., 2016; Zou et al., 2016; Bourlieu et al., 2015a; Gallier et al., 2015b; Lonnerdal, 2014). The safety, ingredient sources, cost and feasibility of these changes in the processing and quality of IF have to be carefully taken into account (Zou et al., 2016;

Delplanque et al., 2015; Lonnerdal, 2014). In order to assure these points and also the appropriate intake of nutrients during infancy, different authorities worldwide are responsible for determining regulations and guidelines for IF, *i.e.* the US Food and Drug Administration (FDA), the European Commission (EC), and the ESPGHAN, as reviewed in Zou et al. (2016).

In IF, **protein components** have generally been added according to HM composition, *i.e.* adjustment of casein/whey ratio, increment of bovine α-lactalbumin, lactoferrin and other bioactive proteins (Wada & Lonnerdal, 2014). Concerns about the protein fraction in IF, primarily based on composition rather than on functional measure, have constantly changed over the decades. For instance, a casein/whey ratio similar to HM was introduced in 1962, but it became widely used only by the 2000s. Regarding protein levels, IF included at first high levels of protein (3.3 – 4.0 g/100 kcal). In the 1960s, renal solute load began to be considered in the design of IF, and lower limits were fixed for protein levels (Committee on the Evaluation of the Addition of Ingredients New to Infant Formula, 2004). However, even if protein content of IF has been lowered during last years, it is still higher than that of HM and leads to significantly higher concentrations of most plasma AA and of blood urea nitrogen than those in breastfed infants (Lonnerdal, 2014). Nowadays, protein intake has been associated to more rapid weight gain during infancy and hence to later obesity (Weber et al., 2014; Koletzko, 2005). EFSA Panel on Dietetic Products, Nutrition and Allergies proposes that IF protein levels should be reduced to 1.8 – 2.5 g/100 kcal, while current regulations preconize a maximum level of 3 g/100 kcal (EFSA, 2014). Beyond the concentrations, it would be important to evaluate if the added bioactive proteins are still able to provide their benefits, since heat treatment can lead to a loss of protein bioactivity (Wada & Lonnerdal, 2015c; Lonnerdal, 2014).

Due to their critical role in infant nutrition, the lipid fraction has been a target considering the IF optimization. In order to get closer to the HM structure, HM fat analogs based on enzymatically or chemically structured lipids with FA regiodistribution mimicking HM have been proposed. Two examples of patented commercial HM fat analogs produced by enzymatic process are Batapol® (1997, US5658768 A) and Infat® (2005, WO2005036987 A1). These analogs, usually called β-palmitate, are suggested to promote several beneficial physiological functions, *i.e.* to positively influence FA metabolism, to increase calcium absorption, to improve bone matrix quality and the stool consistency, to influence the development of the intestinal microbiome (Havlicekova, Jesenak, Banovcin, & Kuchta, 2016). Notably, a special attention is paid to the positional distribution of palmitic acid, which presents substantial benefits for the infant growth when esterified at the *sn*-2 position, as in HM (**Figure 8**) (Zou et al., 2016; Innis, 2011). In despite of these advances, the *sn*-2 position FA distribution of IF is still very different from HM (Innis, 2011). A recent trend in IF development is the production of complex matrices with similar properties to the whole membrane

MFG structure (Zou et al., 2016). For example, a novel IF concept has been developed with phospholipid-coated lipid droplets, Nuturis® (WO2013135739A1) (Gallier et al., 2015a). This IF contains biomimetic droplets presenting comparable size and a phospholipid coating mimicking structural aspects of HM MFG, but the particle size distribution and the membrane MFG composition and organization is again not totally similar to HM. Results from clinical trials studying the use of standard IF enriched with MFG membranes fraction were discussed in section 1.1.5. Another strategy is the reintroduction of cow's milk fat up to 50% of the total lipid. This is promoted by several research groups, including ours (Hernell et al., 2016; Lonnerdal, 2016; Bourlieu et al., 2015a; Delplanque et al., 2015).

Combination of different compounds has been tested as well. Recently, Mudd et al. (2016) proposed a novel IF supplemented with prebiotics (polydextrose and galacto-oligosaccharides), native bovine-derived MFG membrane and lactoferrin. As compared to a control IF, this combined IF supplementation was well tolerated, supported normal growth and positively influenced early postnatal brain development in piglets.

Despite all the improvements implemented to IF, several studies have reported important differences in the short and long-term outcomes of breast-fed compared to IF-fed infants.

1.3. Stabilizing human milk by physical treatments

The Holder pasteurization applied in HM allows a good compromise between microbiological safety and quality of HM. However, various authors have stated an impact of processing (collection, container changes, freeze-thaw cycles) and Holder-pasteurizing HM on its nutritional and biological composition (Peila et al., 2016; O'Connor et al., 2015), microstructure (Deglaire et al., 2013; Lopez et al., 2013), pre-hydrolysis state (Bertino et al., 2013; Bitman et al., 1983), physiological and nutritional outcomes (Andersson et al., 2007; Thomaz et al., 1999; Williamson et al., 1978). These impacts are discussed below in this section, as well as other treatments that could reduce their side effects if applied after or instead of Holder pasteurization (ultrasounds, high pressure, and flash pasteurization).

1.3.1. Impact of Holder pasteurization and associated freeze-thaw cycles

Impact on milk components

Holder pasteurization and the associated double freeze-thaw cycles (**Figure 7**) can lead to varying degrees of nutrient loss, as summarized in **Table 3**.

Several immunological and nutritional components are affected, such as immunoglobulins (IgA, IgG, IgM) (Koenig, de Albuquerque Diniz, Barbosa, & Vaz, 2005; Ford, Law, Marshall, & Reiter, 1977); cytokines (IFN-γ, TNF-α, IL-1β and IL-10) (Ewaschuk et al., 2011); growth factors (insulin-like growth factors, hepatocyte growth factor) (Ewaschuk et al., 2011; Goelz et al., 2009); vitamins (folate, vitamins C and B6) (Molto-Puigmarti, Permanyer, Castellote, & Lopez-Sabater, 2011; Vanzoerengrobben, Schrijver, Vandenberg, & Berger, 1987) and endogenous enzymes (lysozyme, lactoperoxidase, alkaline phosphatase, lipoprotein lipase, BSSL) (Akinbi et al., 2010; Hamprecht et al., 2004; Henderson, Fay, & Hamosh, 1998). However, large variations are observed between studies; this may result from the different time/ temperature to which HM has been exposed, as well as from the different methods of analyzes for compound quantification.

Table 3. Effects of Holder pasteurization on HM components

Component	Maintained (>90%)	Maintained (50–90%)	Maintained (10–50%)	Abolished (<10%)
Macronutrients	Carbohydrate (Lactose, Oligosaccharides)	Protein Total fat		
Micronutrients	Calcium Copper Magnesium Phosphorus Potassium Sodium Zinc	Iron		
Vitamins	Vitamin A	Folate Vitamin B6 Vitamin C		
Biologically active (immune)	IL-8, IL-12p70, IL-13 TGF-α	IgA, sIgA IgG IGF-1, IGF-2 IGF-BP2,3 IFN-γ IL-1β, IL-4, IL-5, IL-10 TGF-β Gangliosides	CD14 (soluble) Il-2 Lactoferrin-iron binding capacity Lysozyme	IgM Lymphocytes
Biologically active (metabolism)	Epidermal growth factor Heparin-binding growth factor	Adiponectin Amylase Insulin	Erythropoeitin Hepatocyte growth factor	Bile salt-dependent lipase Lipoprotein lipase

Ig, immunoglobulins; IGF, insulin-like growth factor; IFN-γ, interferon- γ; IL, interleukin; TGF, transforming growth factor. Source: O'Connor et al. (2015)

Concerning macronutrients, the protein fraction is suggested to be affected both in terms of concentration and functionality (Peila et al., 2016; O'Connor et al., 2015; Ballard & Morrow, 2013). Notably, lactoferrin concentrations and its iron-binding capacity are reduced by as much as 60% (Akinbi et al., 2010; Ford, Law, Marshall, & Reiter, 1977). The apparent decrease in lactoferrin concentrations is likely to be due to heat denaturation and formation of carbonyls, with a subsequent formation of disulfide-bonded high molecular aggregates, which then prevents its quantification in chromatographic analyses (Baro et al., 2011). Conversely, the concentrations of α-lactalbumin and serum albumin are not significantly affected by pasteurization (Akinbi et al., 2010).

García-Lara (2013) reported an average decrease of 6.2% of fat after pasteurization, which could result of an increase in the concentration of oxidized FA and derivatives that cannot be measured by the HM analyzer. Similarly, Wardell et al. (1981) reported a loss of LCPUFA by oxidation during freeze-thaw and pasteurization. However, Henderson et al. (1998), Michalski et al. (2008) and Ewaschuk et al. (2011) showed no impact of pasteurization in these LCPUFA, potentially due to the high antioxidant activity of HM. Overall, the persistence of this oxidant activity after pasteurization is controversial. Some authors reported a decreased antioxidant capacity in HM after pasteurization, as reported by Silvestre et al. (2008) after measurement of glutathione concentration and glutathione peroxidase activity. Conversely, Elisia & Kitts (2011) found no changes in this activity, suggesting that no lipid oxidation occurred during Holder pasteurization (as measured by hexanal level, ORACFL and malondialdehyde content). More studies are needed on the assessment of the oxidative stability of the pasteurized HM, especially as it is stored over long periods in HMB.

Impact on milk structure

Heat treatments together with freeze–thaw cycles can induce the disruption of the MFG membranes (Vieira, Soares, Pimenta, Abranches, & Moreira, 2011; Wardell, Hill, & Dsouza, 1981), protein denaturation and some aggregation of proteins in the soluble phase and on the MFG membrane leading to thicker globule interfaces (Deglaire et al., 2013; Raikos, 2010; Ye, Singh, Taylor, & Anema, 2004). However, data on HM are still limited and available data concerns mainly bovine milk. As bovine milk and HM have a similar general emulsion organization, they are often assumed to behave similarly when submitted to physical treatments. Nevertheless, the differences should not be underestimated since the properties of oil-in-water emulsions depend on the interaction between droplets and the types of protein adsorbed to their surface (Dalgleish, 2006).

Previous work from our team confirmed that likewise to bovine milk, Holder pasteurization of HM induces aggregation of protein in the soluble phase and around the human MFG membrane (Deglaire et al., 2013). However, more studies are necessary to describe the impact of physical treatments on the molecular configuration and functionality of different components of HM.

The disruption of the MFG membranes may facilitate coalescence and thus the adherence of fat, associated proteins and other nutrients to the walls of delivery devices and mainly to the feeding tubes during the enteral nutrition (Borgo, Coelho Araujo, Conceicao, Sabioni, I, & Mendonca, 2014; Chang, Chen, & Lin, 2012). The nutrient (and mainly fat losses) during the steps of processing and delivering HM is a problem which have been raised by several authors who have proposed strategies to reduce these losses, *i.e.* optimization of pre-exposure manipulation and adaptation of infusion systems (Jarjour et al., 2015; Rayyan et al., 2015; Garcia-Lara et al., 2014).

Impacts on human milk digestion and nutritional outcomes

Since Holder pasteurization severely affects the activity of a large number of endogenous enzymes and anti-proteases naturally present in HM (**Table 3**), it may modify the natural equilibrium between the pre-digestive and anti-digestive processes that occur in expressed HM, as described in section 1.1.3. In particular, because of the compensatory function of endogenous lipases for digestion in newborns (Bourlieu et al., 2014; Hamosh, 1994), pasteurization of HM will potentially affect its gastrointestinal digestion. In addition, the heat-induced protein denaturation and aggregation on the MFG membrane could also modulate the enzyme access and the HM's susceptibility to digestion (Bourlieu et al., 2015a; Raikos, 2010).

There are few studies comparing raw and pasteurized HM-feeding with HM having similar composition. Williamson et al. (1978) studied seven preterm infants receiving the same pool of HM from several donors as raw (after a freeze-thaw cycle), pasteurized or boiled. Each of the three milks was fed to each infant for one of three consecutive weeks, in a different order. The authors reported higher growth rate and fat absorption during the week infants were fed raw HM as compared to pasteurized and boiled HM; no difference was observed for absorption of nitrogen, calcium, phosphorus and sodium. The mean weight gain during raw HM feeding was approximately one-third greater than that during pasteurized HM feeding. Similarly, Andersson et al. (2007) studied five preterm infants fed their mother's own milk as raw or pasteurized. They were randomized in two groups, of which one started with pasteurized milk for one week and continued with raw milk the following week, and a second group was fed in reverse order. The authors observed higher growth rate and higher coefficient fat absorption (17% higher) during the week infants were fed HM.

These findings were attributed to the heat inactivation of BSSL by the Holder pasteurization, thus inducing a decrease in fat digestibility in the newborn. Another explanation could be the nutrient losses by adherence inducing a lower energy and nutritional supply, which is more susceptible to happen during pasteurized compared to raw HM-feeding (Borgo et al., 2014; Vieira et al., 2011).

Recently, Wada & Lonnerdal (2015b) compared the patterns of peptides released from major proteins in raw and Holder-pasteurized HM after *in vitro* digestion. The authors suggested that pasteurization of HM did not affect the formation of bioactive peptides to a significant extent. However, the HM samples were obtained from one unique volunteer mother and digested in a static *in vitro* system in which exclusively proteolytic enzymes were used.

In summary, although the impact of Holder pasteurization on the HM components is well established, its impact on HM digestion remains unclear and should be further investigated.

1.3.2. Homogenization of pasteurized human milk

In order to reduce the side effects of Holder pasteurization, some authors have proposed the application of a second treatment on the pasteurized HM: the homogenization by ultrasonication (Thomaz et al., 1999; Rayol et al., 1993). The ultrasounds process applied to liquids is based mainly on the cavitation phenomenon, which creates a combination of pressure, heat and turbulence through cycles of rarefaction and compression when ultrasound is propagating (Soria & Villamiel, 2010). In the different studies in which sonication of HM is conducted, the methodologies vary mainly in terms of power (from 25 to 200 watts), time (from 90 s to 15 min) and type of homogenization (direct or indirect) (Christen, Lai, & Hartmann, 2012; Thomaz et al., 1999; Dhar, 1996; Rayol et al., 1993; Martinez, Desai, Davidson, Nakai, & Radcliffe, 1987).

Homogenization results in the fragmentation of the native MFG (about 4 µm) into smaller and uniformly distributed lipid droplets (from 0.1 to 1.5 µm), with the size distribution depending on the ultrasonic power and length of sonication (Ashokkumar et al., 2010; Dhar, 1996). Hence, similarly to high-pressure homogenization, the specific surface area of lipid droplets is significantly increased (Garcia, Antona, Robert, Lopez, & Armand, 2014). **Figure 9** shows particle size distribution of the same milk as raw, pasteurized and pasteurized + homogenized (Bellanger, 2014). In this case, ultrasonic homogenization was performed using a Q700 Sonicator (QSonica, Newtown, USA) at a power of 595 W (three periods of 5 min interrupted by 30s of pause).

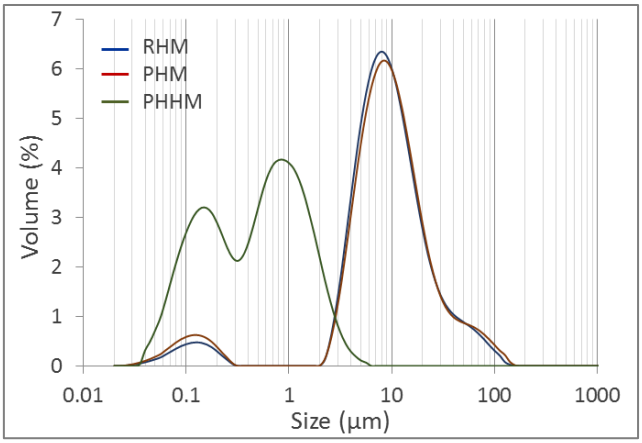


Figure 9. Particle size distributions of human milk either raw, pasteurized or pasteurized-homogenized (RHM, PHM and PHHM, respectively). Determined by laser light scattering in EDTA. N = 1 anonymous donor. Adapted from: Bellanger (2014).

In bovine milk, the neo-formed lipid droplets are stabilized by fragments of the native membrane and milk proteins including heat-denatured whey proteins, casein micelles and fragments of casein micelles (Garcia et al., 2014; Michalski & Januel, 2006). These proteins adsorbed at the surface of the droplets form a thick layer, which reduces the interfacial tension and prevent droplets from

coalescence or flocculation via steric and electrostatic stabilization mechanisms (Raikos, 2010). In addition, casein micelles may also be disrupted (Michalski & Januel, 2006). Other nutrients such fat-soluble vitamins and minerals are usually bound to the formed droplets (Bermudez-Aguirre, Mawson, & Barbosa-Canovas, 2008).

The increase in the specific surface area is assumed to facilitate enzyme adsorption, as observed during gastric infant *in vitro* digestion of bovine milk emulsions (Bourlieu et al., 2015b). Concerning proteins, the same study reported a faster proteolysis after homogenization and hypothesized that the adsorbed proteins were more susceptible to gastric digestion than solubilized proteins (Bourlieu et al., 2015b).

Previous studies have reported significant increases in fat absorption (Thomaz et al., 1999) and in weight gain (Rayol et al., 1993) in preterm newborns fed pasteurized and homogenized HM compared to pasteurized HM. This can be explained by an improved fat digestion and/or to a reduction of nutrient losses by adherence to the walls of the bottle and feeding system (Thomaz et al., 1999; Rayol et al., 1993; Martinez et al., 1987). However, there is no study about the effect of the HM homogenization on its digestion.

1.3.3. Alternative treatments to Holder Pasteurization

Some alternative to Holder pasteurization (flash pasteurization, high pressure, ultrasounds) have been proposed to ensure elimination of pathogens but preserving as much as possible bioactive components of HM.

The thermosonication (or ultrasonic pasteurization) consists in the synergic effect of ultrasounds with a thermal treatment, which would be able to reduce the treatment time and energy (Christen et al., 2012; Dhar, 1996). In the same time, it leads to the homogenization of the HM with the disruption of the native MFG. Dhar (1996) proposed the use of thermosonication for rapidly treating small batches of HM (90 s, 80 mL) in constant agitation, which provided high preservation of immunoglobulins and prevented fat loss during tube feeding. Accordingly, Czank et al. (2010) reported that thermosonication of HM was more effective than Holder pasteurization on retaining IgA (91%), lysozyme (80%), lactoferrin (77%) and BSSL (45%). However, there are some controversial findings regarding the efficacy of thermosonication on ensuring the microbiological safety of milk, as the power necessary for attending that results in increase of temperature and hence protein denaturation (Christen et al., 2012; Dhar, 1996).

The flash pasteurization is a high temperature short time method (72 °C, 15 s) often applied in the dairy industry. Recently, some authors reported that the flash pasteurization applied to HM is more effective than the Holder method preserving the integrity and the activity of BSSL, and in some

extent the protein profile, mainly regarding the native form of lactoferrin and IgA (Giribaldi et al., 2016; Baro et al., 2011). However, even if higher after flash pasteurization, the BSSL activity found by Giribaldi et al. (2016) (0.26 ± 0.10 U/mL) was much lower than that found by Baro et al. (2011). Results from Giribaldi et al. (2016) are coherent with the well-established high sensibility to heating of BSSL (Henderson, Fay, & Hamosh, 1998). Furthermore, in contrast to these two studies, other authors have found a reduction of immunological components after flash pasteurization (Chantry et al., 2009; Dhar, Fichtali, Skura, Nakai, & Davidson, 1996). This variation in data regarding retention of proteins following different pasteurization methods is likely due to differences in methods of analysis, the milk volume processed, conditions of process before and after the heat treatments, etc. (Dhar et al., 1996).

Among the non-thermal process, the high-pressure technology has been increasingly applied in food industry as an alternative to thermal treatments. Based on a principle of compression and uniform pressure from every direction, this treatment damages the cell membranes present in the liquid without significantly affect nutrients and bioactive compounds (Sousa et al., 2016). Few studies were performed for evaluating the effects of high-pressure processing of HM on its overall microbiology safety and nutritional quality.

Ultraviolet irradiation is another non-thermal process, based on electromagnetic radiation. Ultraviolet-C, specifically a wavelength between 250 and 270 nm, is reported to be highly germicidal. Christen et al. (2013a; 2013b) investigated the bacterial content and the preservation of some components of HM after application of the ultraviolet-C irradiation compared to Holder pasteurization. The authors found that this method was capable of assuring the microbiologic safety of HM and the retention of IgA, lactoferrin and lysozyme was respectively 89%, 87% and 75%, whereas the Holder method retained respectively 49%, 9% and 41% (Christen, Lai, Hartmann, Hartmann, & Geddes, 2013a). Furthermore, compared to fresh HM they found no significant changes in the FA profile, BSSL activity or alkaline phosphatase activity after the application of ultraviolet-C irradiation up to the dosage required for a 5-$\log_{10}$ reduction of the five species of bacteria (Christen, Lai, Hartmann, Hartmann, & Geddes, 2013b). Nevertheless, ultraviolet-C irradiation is a known generator of reactive oxygen species, which potentially leads to imbalanced oxidative status and damages HM components if present in excess (Christen et al., 2013a). The generation of these components and their effect on HM components was not directly investigated.

In summary, studies about alternative treatments with minimal impact on the HM properties have continuously been performed. Alternative methods to Holder pasteurization could help preserving HM nutritional and immunological properties, but still have to be extensively evaluated and fully validated for the technical and safety standards. Moreover, further studies are necessary to adapt

new techniques to the routine of HMB and neonatal care units, which is completely different from the reality of dairy industries or laboratory scale.

1.4. The developing digestive system

The digestion process comprises chemical, mechanic and enzymatic mechanisms and involves successive steps (oral, gastric and intestinal digestion). The digestive conditions and efficacy depend on many factors, notably on the physiological stage (infant, adult, elderly) and on the characteristics of the food ingested. Although the development of the digestive functions occur early during the uterine life, newborn infants (< 28 days of life) and infants up to six months are recognized to have an immature digestive system compared to older infants (> 6 months). The underdeveloped digestive function of newborn infants can have an impact on their ability to digest and absorb HM nutrients, compromising their adequate growth and development (Poquet & Wooster, 2016; Bourlieu et al., 2014).

This section presents the key parameters of the digestion of milk-based diet by term and preterm newborn infants, including physiological characteristics, enzymatic processes and emulsion disintegration. We will focus mainly on the gastroduodenal digestion of lipids and proteins. Finally, digestion models for studying infant digestion will be briefly discussed.

1.4.1. Newborn infant digestion

Some characteristics of the developing digestive system of the infant are summarized in **Figure 10**. Digestive immaturity at birth is mainly linked to secretory functions, motility and enzymes. In particular, newborn infants have low pancreatic enzyme and submicellar bile salt concentrations (Poquet & Wooster, 2016; Bourlieu et al., 2014). Therefore, choosing a diet that respects these constraints is essential to ensure the development of the gastrointestinal tract and its functions. Thus, infants are fed **exclusively on liquid milk-based diet** during the first months of life (ideally HM).

In infants receiving an exclusive liquid diet, the oral phase slightly participates on the digestive process, although the swallowed saliva can interact with the meal during the gastric phase (glycoprotein and salivary amylase mainly) (Bourlieu et al., 2014). Up to 80% of developmentally delayed infants (including preterm infants) experience oral feeding difficulties due the immaturity of physical and neurophysiologic functions, resulting in no synchronous activities of sucking, swallowing, breathing, and esophageal function (Lau, 2015). The disability to breast- or bottle-feed safely and efficiently leads to the prescription of enteral nutrition.

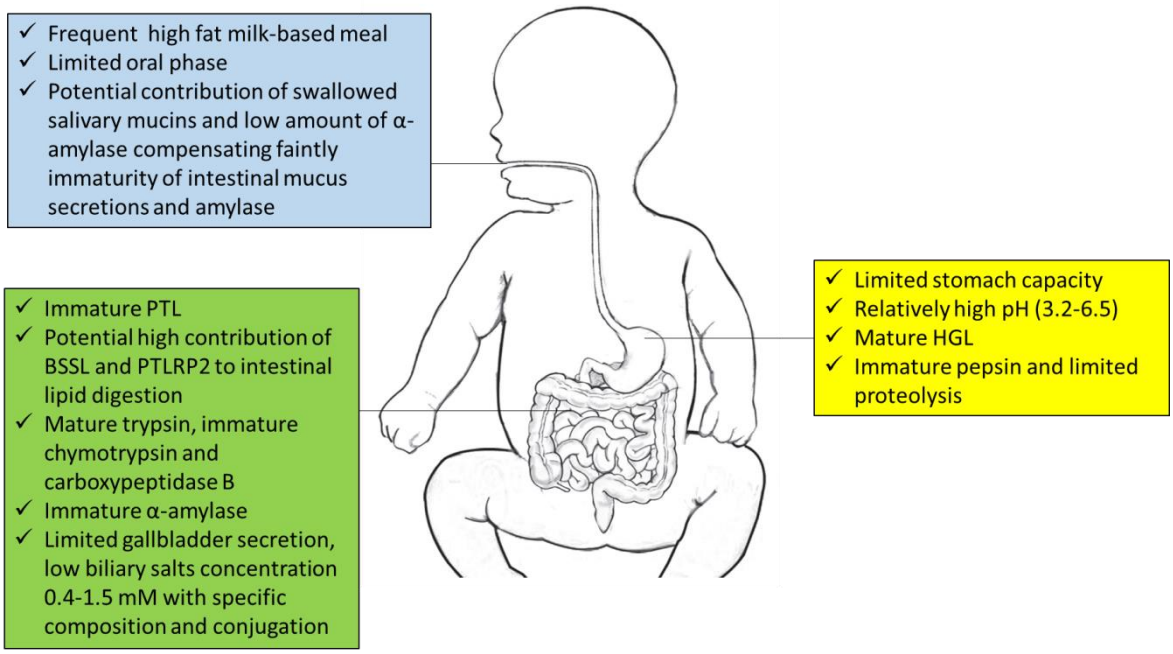


Figure 10. Summary of the developing digestive physiology in the infant. Adapted from: Shani-Levi et al. (2017). BSSL, bile salt-stimulated lipase; HGL, human gastric lipase; PTL, human pancreatic triglyceride lipase; PTLRP2, pancreatic triglyceride lipase-related protein 2.

The stomach is the first main digestive compartment in the infant. This compartment stores, mixes, initiates the hydrolysis and regulates the emptying of milk bolus from the stomach into the small intestine. Gastric juice, secreted by the epithelial cells of the gastric mucosa, contains mainly enzymes (pepsinogen, HGL), hydrochloric acid, sodium bicarbonate as well as mucins that form a protective barrier for the gastric mucosa (Geigy, 1973). The chyme (meal and gastric secretions) progressively arrives into the small intestine, which is separated into three parts: duodenum, jejunum and ileum. This phase is more marked by the immaturity of digestive functions during the neonatal period than the gastric phase; however, most of the hydrolysis and absorption of nutrients takes place in duodenum and jejunum (**Figure 10**). The next step, the large intestine, is mostly in charge of the absorption of water and electrolytes.

Some accessory organs are crucial to the digestive process, *i.e.* pancreas (production of the alkaline pancreatic secretion containing lipases, proteases and amylase), liver (production of bile, metabolism of lipids and proteins, storage of glycogen and vitamins) and gallbladder (storage and release of bile toward the duodenum) (Berseth, 2006).

For all the mentioned steps, preterm newborn infants have their digestive capabilities still more affected than term infants. **Figure 11** illustrates this difference between term and preterm stages regarding the maturity of the main enzymes acting in gastric and intestinal digestion. Notably, the efficacy of the enzymatic hydrolysis and of absorption are affected by several factors such as pH, lumen mineral content (*e.g.* Ca^{2+} precipitation of FA), and the presence of biosurfactants (*e.g.* the

role of bile salts and phospholipids in FA and MG absorption). These factors, on their turn, change through digestion due to gastric or pancreatic secretions (Poquet & Wooster, 2016).

Regarding the digestion of carbohydrates, lactose is hydrolyzed to galactose and glucose by lactase in the small intestine. Glucose is transported via the hepatic portal vein to the liver before passing into the peripheral circulation and be available for energy production. The digestion of lipids and proteins will be commented bellow in separated topics.

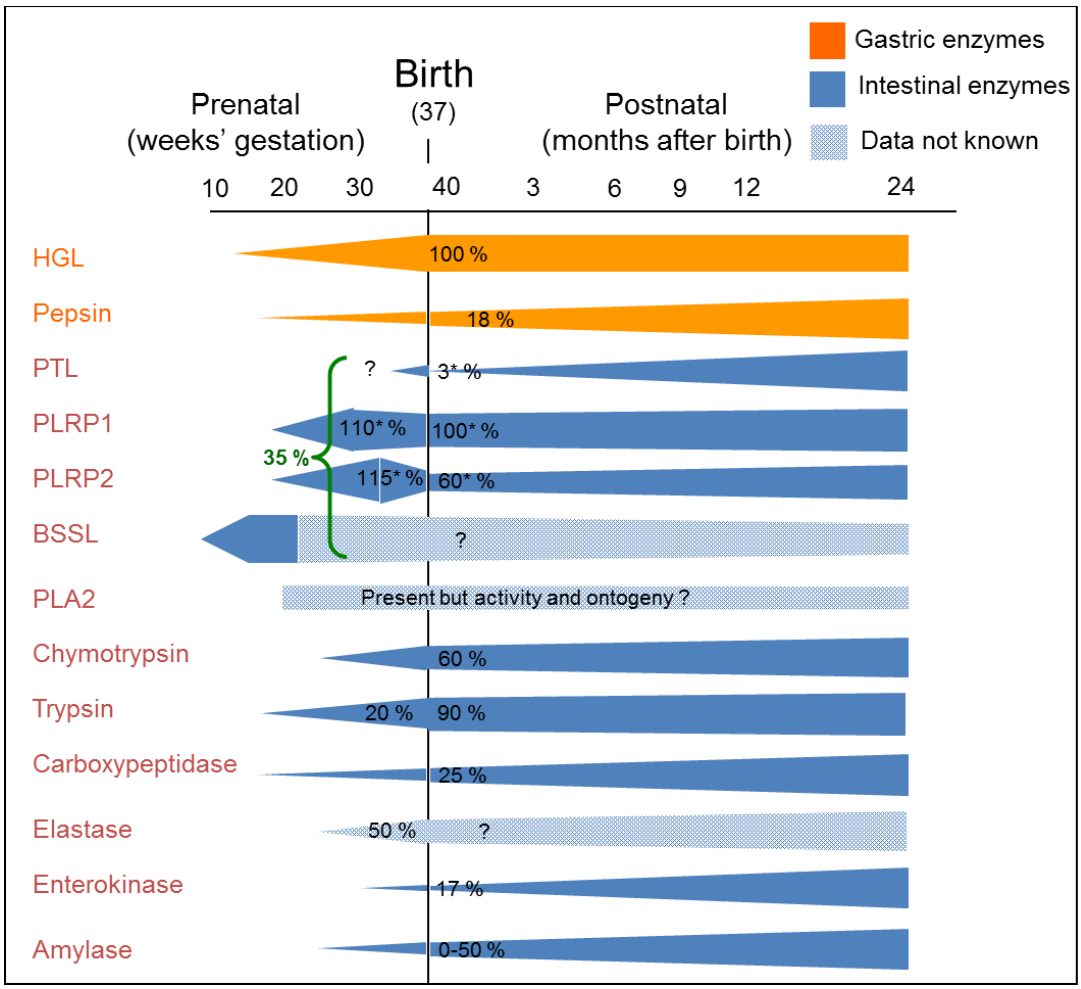


Figure 11. Ontogeny, levels of activity or of mRNA expression (*) of the main digestive enzymes. Figure refers to the values at birth or at a given postnatal age for term infants. Source: Bourlieu et al. (2014).

1.4.2. Gastric phase: a key step for infant digestion

Gastric phase is considered a key step for newborn infant digestion, especially in the preterm stage. Indeed, compared to older infants, protein gastric digestion by pepsin is lower due to lower proton pump activity (and relatively high pH, *i.e.* 6 – 5 during the first hour of digestion) and lower pepsin levels. Furthermore, the importance of HGL to fat digestion is higher in the newborn due to immature intestinal lipolytic capacity (Lindquist & Hernell, 2010; Hamosh, Henderson, & Hamosh, 1998). Thus, gastric kinetics of lipolysis and proteolysis play a major role determining in which state

nutrients will arrive in the small intestine and then influencing the intestinal kinetics of nutrient hydrolysis, absorption and bioactivity functions. Finally, the intestinal kinetics may orient metabolic pathways of AA and FA after absorption, as previously demonstrated in adults (Vors et al., 2013; Deglaire et al., 2009; Lacroix et al., 2006).

The output and the composition of gastric fluids are influenced by hormones (cholecystokinin, gastrin, secretin) and by other factors (acidity, mixing, gastric emptying rate). In the fasting state, gastric secretions and residual of the precedent meal may be present in the stomach (Bourlieu et al., 2014). After ingestion, the gastric emptying rates directly influence postprandial events such as pH and enzymes concentration. When the meal leaves the stomach, the ratio meal to secretions reduces and then the pH decreases. Thus, besides the enzymes secretion and level, gastric digestion is governed by the dynamic of emptying rate and pH decrease (Sams, Paume, Giallo, & Carrière, 2016).

The **gastric emptying** depends on several factors linked to milk composition and structure, infant gestational and postnatal ages. Furthermore**, hormonal feedback** may also govern the contractions of the antrum and pylorus, and then the gastric emptying. The gastric emptying pattern is reported to be exponential and accurately fitted using Elashoff model (Elashoff, Reedy, & Meyer, 1982):

$$f = 2^{-(t/t_{1/2})^{\beta}} \qquad (1)$$

with *f*, fraction remaining in stomach at a given time *t*, *T1/2*, the time from the start of the meal until 50% of the meal has been emptied also (gastric half-emptying time), β coefficient determining the shape of the curve and the intensity or lack of initial lag time.

Although large variations are reported in the literature, it is well accepted that the gastric emptying is slower for term than for preterm infants, and for HM than for IF (Bourlieu et al., 2014). As an example, experimental data from the literature were fitted for these different stages and meals using the Elashoff model, as presented in **Figure 12**A.

Regarding the **gastric pH decrease**, most part of the available data concerns preterm infants, while only Mason (1962) studied the gastric pH decrease in term newborns. The pH decrease during newborn gastric digestion does not reach adult levels due to two main reasons: immaturity of acid secretion and high buffer capacity of milk-based meals. Thus, the acidification capacity of the newborn seems not depend on the type of meal (HM *versus* IF) (Bourlieu et al., 2014; Armand et al., 1996). Contrary to the emptying rate, gastric pH decrease seems to follow a **linear regression** (**Figure 12**B).

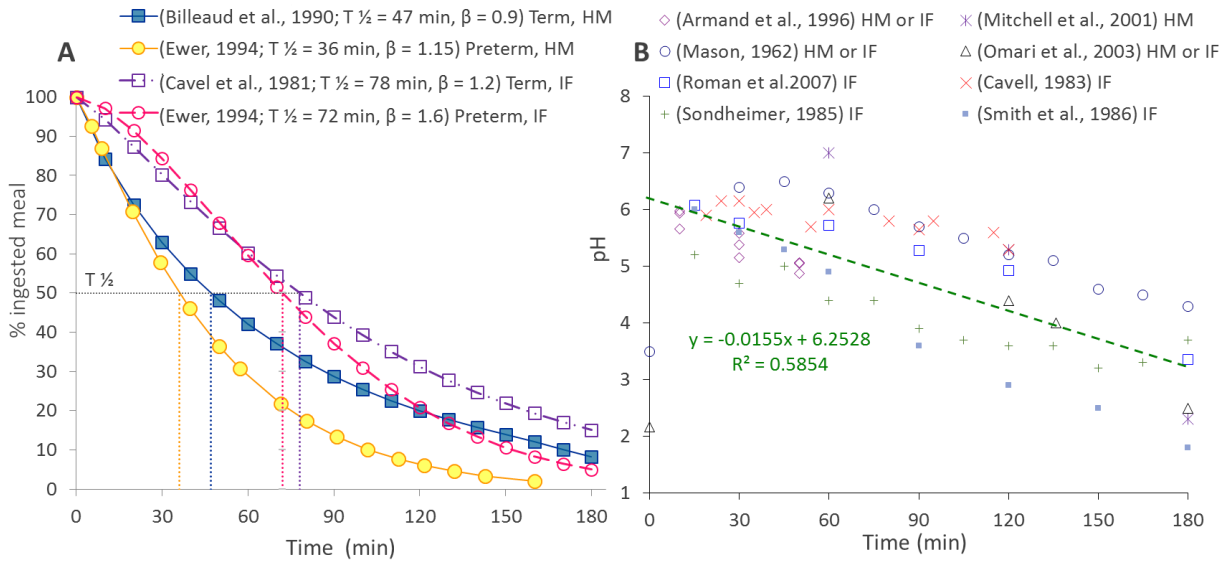


Figure 12. Gastric emptying rate fitted from experimental data using Elashoff model (A) and gastric pH decrease as reported in *in vivo* studies (B). Data adapted from Bourlieu et al. (2004).

1.4.3. Lipid digestion

The efficient digestion and absorption of fat is a multistage process depending on different enzymes (Bernback et al., 1990).

In HM, endogenous enzymes (*i.e.* BSSL) start lipids digestion, as discussed in section 1.1.4. The pre-lipolysis level in undigested HM is reported to be limited and it is not even measured in most of studies dealing with gastrointestinal lipolysis, which consider the HM pre-lipolysis level as equal to zero (Roman et al., 2007; Armand et al., 1996). Thus, its impact on the gastrointestinal lipolysis of HM remains unclear (Bourlieu et al., 2014; Bertino et al., 2013; Pamblanco et al., 1987). Contrary to other species, in humans there is no pre-gastric lipase (lingual lipase in rodents for instance) contributing to lipid digestion (Carrière, 2013).

In the **gastric phase HGL** will hydrolyze mainly TG, presenting a preference for *sn*-3 position FA and an optimal pH of 5 to 5.4 (Carrière et al., 1997). This stereo-selectivity results in an apparent specificity for SC and MCFA, which can be absorbed directly *via* the gastric mucosa as free FA (Lemarie, Beauchamp, Legrand, & Rioux, 2016; Roman et al., 2007; Bernback, Blackberg, & Hernell, 1989). In general, HGL is mature in both term and preterm newborns, hydrolyzing between 10 and 30% of TG. Gastric lipolysis is reported to be 1.7 to 2.5 times higher in HM than in IF-feeding (Lindquist & Hernell, 2010; Roman et al., 2007; Armand et al., 1996). Even if considered limited, gastric lipolysis likely compensates for the pancreatic insufficiency regarding lipase production, since the release of free FA from gastric lipolysis promotes the activation of the HPL–colipase complex (Michalski, 2009). Indeed, it was demonstrated that initial TG digestion by HGL is necessary for

efficient fat digestion by HPL and/or BSSL in the intestinal phase, leading to complete digestion of TG (free FA and glycerol as final products) (Bernback et al., 1990).

In the duodenum, lipases secreted by the exocrine pancreas will complete the digestion of HM fat, together with bile salts. Other lipases also present in the intestinal phase are: the pancreatic phospholipase A2 (PLA2), pancreatic lipase related-proteins 1 and 2 (PLPR1 and 2) and BSSL (or CEH). PLA2 hydrolyzes phospholipids, stereospecific for the *sn*-2 position. PLPR1 has an activity on TG and PLPR2 seems to complement the action of HPL, but their function on infant digestion is not well established (Berton, Sebban-Kreuzer, Rouvellac, Lopez, & Crenon, 2009). HPL is the major pancreatic lipase in adults, hydrolyzing mainly TG in the *sn*-1,3 positions and leading to release of *sn*-2 MG and free FA. HPL needs a colipase to be active in the presence of bile salts and is sensitive to the degree of emulsification. BSSL is a non-specific and non-regio-selective enzyme, being able to hydrolyze TG, partial glycerides (including the *sn*-2 MG released by the HPL), cholesterol ester and fat-soluble vitamin esters in all three ester-bonds (Bourlieu et al., 2014; Hernell & Blackberg, 1994).

In brief, each lipase contributes with its specific properties. The lipolysis products resulting from milk fat digestion are mainly free FA and *sn*-2 MG. The main steps involved on intestinal fat absorption are solubilization by bile salts, uptake of the free FA through the brush border, transport within the enterocyte, re-esterification into TG or phospholipid, incorporation into chylomicrons and release from the enterocyte through the lymphatics into the circulation. SCFA and some MCFA diffuse passively across the enterocyte membrane and are taken up as free FA (bound to albumin) into the liver through the portal vein (Hamosh, 2006). LCFA, because of their lipophilic nature, will enter the cells carried by diffusion mechanism (mixed micelles) or protein-mediated uptake model (FA bind to FA transport proteins) (Poquet & Wooster, 2016). However, if not solubilized in micelles some LCFA may be precipitated into insoluble calcium soaps and excreted in stool, reducing the absorption of both calcium and LCFA (Gallier, Acton, Garg, & Singh, 2016a; Michalski, 2009).

Beyond the enzymatic hydrolysis, bile salts excreted in the intestinal lumen play an essential role in the fat hydrolysis, absorption and transport. They are crucial for the emulsification of the chyme, the solubilization of lipolysis products into an absorbable form (notably LCFA) and for triggering the action of HPL/colipase and BSSL. However, in postprandial conditions, the chyme arriving in the duodenum dramatically reduces the baseline bile salts concentration to around 1 mM, which is lower than the critical micellar concentration required for solubilization of the products of lipid digestion and its subsequent absorption, *i.e.* approximately 4 mM (Poquet & Wooster, 2016; Bourlieu et al., 2014). In adults, levels of up to 30 mM can be reached after a fatty meal (Abrahamse et al., 2012).

The amount of fat that is excreted with the stool appears to depend essentially on gestational and postnatal age and on the type of fat. The coefficient of fat absorption ranges from 74 to 91% in

preterms after ingestion of HM, 68 to 85% in preterm and around 90% in term infants after ingestion of IF, and generally > 95% in healthy adults (Lindquist & Hernell, 2010). This limited coefficient of fat absorption in infants is likely to be linked to the maturation of the digestive system, of the digestive function (*i.e.* enzyme and bile salt levels), of the absorption capacity, or a combination of all (Abrahamse et al., 2012).

Since the production of pancreatic BSSL, HPL and bile salts is immature in newborns, the HM carrying BSSL and bile salts may help to complete the intestinal fat digestion via distinct mechanisms. First, BSSL plays a crucial role in fat absorption by increasing the release of free FA, which are more readily absorbed in the typical conditions of low concentration of bile salts (*i.e.* poor capacity of micellar solubilization, transport and absorption of MG) (Bernback et al., 1990). Second, the bile salts present in HM (Forsyth et al., 1990) may contribute to both BSSL activation and the formation of micelles. Furthermore, the emulsifying properties of the MFG membrane rich in phospholipids also contribute to the formation of micelles, which is a specific contribution of the lipid architecture in HM to the fat digestion (Abrahamse et al., 2012).

1.4.4. Protein digestion

Enzymes hydrolyzing proteins are classified as endo- or exopeptidases. Endopeptidases cleave peptide bonds at the middle of the protein sequence, releasing large peptide fragments that will be subsequently hydrolyzed to small peptides. Exopeptidases cleave the peptide bond at the N-terminal (aminopeptidases) and C-terminal (carboxypeptidases) releasing the terminal AA one by one. The combined action of these enzymes enables the degradation of proteins into peptides and free AA (Rassin & Shattuck, 2006).

Proteolysis in the stomach is catalyzed by pepsin, an endopeptidase activated from pepsinogen by selective cleavage of a small basic peptide. The secretion of pepsinogen in newborn infants is reported to be lower than in children and adults, reaching adult levels by only two years of age (Figure 11) (Henderson, Hamosh, Armand, Mehta, & Hamosh, 2001; Hamosh et al., 1998). Furthermore, the relatively high gastric pH in newborn is far from the pH optimum of pepsin, *i.e.* pH 2 (Piper & Fenton, 1965). Thus, the combination of these two factors justifies the limited proteolysis reported during the gastric phase (Chatterton et al., 2004; Henderson et al., 2001). The major gastric proteolysis products are polypeptides with N-terminal AA including phenylalanine and leucine, as well as small amounts of oligopeptides and AA.

In the intestinal phase, pancreatic proteases are secreted as inactive zymogens, which are activated by trypsin; trypsin is activated from trypsinogen by intestinal enterokinase, secreted from intestinal epithelial cells in response to food stimulation (Bourlieu et al., 2014; Dallas et al., 2012). *In vivo* data

presents high variability in the proteolytic activity in the intestine, with the ontogeny of trypsin and chymotrypsin in one-month old preterms corresponding to around 90% and 60% of the level found in adults, respectively (Bourlieu et al., 2014). Trypsin cleaves peptides at the carboxyl side of lysine and arginine, while chymotrypsin cleaves on the carboxyl side of tyrosine, tryptophan or phenylalanine. Complementing their action, carboxypeptidase B cleaves the basic AA arginine and lysine from the C-terminal (Dallas et al., 2012). Other peptidases in the intestinal brush border (*e.g.* γ-glutamyltranspeptidase, oligoaminopeptidase, dipeptidylaminopeptidase IV) may help to complete the hydrolysis of peptides into AA, even in premature infants. The peptides and free AA generated are absorbed by carrier-mediated mechanisms across the basolateral membrane into the portal blood, by intracellular vesicle mediated transport system (transcytosis) or by paracellular passive diffusion (Wada & Lonnerdal, 2014).

Like lipids, the susceptibility of proteins to digestion depends on specific structure characteristics and on their organization in HM (Zhang et al., 2014; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009). Factors like phosphorylation, size, charge, tertiary structure, AA content and glycosylation are suggested to influence degradation of proteins (Dallas et al., 2012). For instance, apo-lactoferrin seems to be more susceptible to digestion than its holo form (Brines & Brock, 1983). Zhang et al. (2014) proposed that HM proteins solubilized in the serum or adsorbed in casein micelles are more accessible for gastric digestion than proteins in immune cells or in the MFG membrane. In bovine milk, Macierzanka et al. (2009) demonstrated *in vitro* that proteins in solution (β-caseins and β-lactoglobulin) were hydrolyzed slower than that adsorbed, likely due to the adsorption-induced changes in the conformation and flexibility of these proteins.

HM proteins such as lactoferrin and immunoglobulin A are found intact in the stools of infants fed HM (Goldman et al., 1990; Schanler, Goldblum, Garza, & Goldman, 1986). The incomplete breakdown of proteins can be harmful (*i.e.* inducing allergic response, limiting AA availability) or benefic depending on the specific protein or peptide remaining intact (Dallas et al., 2012). Indeed, the global impaired HM proteolysis is often considered benefic since some intact proteins and peptides exert several bioactive functions directly in the gastrointestinal lumen or at peripheral organs after being absorbed at the intestinal mucosa (Lonnerdal, 2016; Labbok et al., 2004). Knowledge about how HM protein are broken down in the digestive tract have been improved by the study of the peptides released and their bioactive functions (Wada & Lonnerdal, 2015b; Dallas et al., 2015; Dallas et al., 2014; Zhang et al., 2014).

1.4.5. Emulsion disintegration

The structure of the milk emulsion, notably the size of MFG and solubility of proteins, are reported to modulate the digestive emulsion behavior and hence the kinetics of lipolysis and proteolysis, as well the nutrients absorption (Bourlieu & Michalski, 2015; Bourlieu et al., 2015b; Berton et al., 2012; Macierzanka et al., 2009; Singh, Ye, & Horne, 2009).

How milk emulsion droplets interact with each other and respond to different environmental conditions depend on the emulsion properties (*i.e.* size of MFG and quantity of interface, type and organization of proteins) and on characteristics of the interfacial layers (*i.e.* thickness, composition, charge) (Gallier et al., 2016; Bourlieu & Michalski, 2015; Singh et al., 2009). These characteristics can differ among milks (*e.g.* HM and bovine milk) and are modified after physical treatments (section 1.3). Thus, the different milks that can be chosen for nourish infants are likely to have different emulsion disintegration patterns during digestion (Bourlieu et al., 2015b; Gallier et al., 2013b; Gallier et al., 2013a; Salentinig, Sagalowicz, & Glatter, 2010; Armand et al., 1996). However, how the structural properties of emulsions change during their passage through the gastrointestinal tract remains little known.

Once in the infant stomach, the milk emulsion is exposed to decrease on pH (generally 6 – 5 during the first hour), mechanical agitation because of peristaltic movements and mix with gastric juices containing enzymes and minerals. Thus, several structural changes may occur, such as creaming, flocculation and coalescence of lipid droplets (physical processes), modification of interfacial layers and hydrolysis (chemical processes), with aggregation and destabilization of the emulsion (Gallier et al., 2016; Singh et al., 2009). Indeed, the gastric acidification can lead to changes in the conformation and charge of proteins, removing electrostatic charges and favoring the flocculation (Golding et al., 2011).

The products of gastric hydrolysis (*e.g.* peptides, free FA, partial glycerides) and other endogenous products (mucins, enzymes) are surface active, influencing the droplet stability (Golding et al., 2011). For instance, the lipolytic products accumulate at the oil–water interface and lead to emulsification of fat droplets. Proteolysis, even if limited, induces a loss of charge on the droplets surface and reduction of the adsorbed layer. It appears to be a major factor leading to emulsion destabilization during the gastric digestion in infants (Bourlieu et al., 2015b; Singh, 2011).

Once arrived in the duodenum, where the pH is neutralized by the secretion of sodium bicarbonate (around 6 -7), the products of gastric hydrolysis will participate in lipid emulsification (Berton et al., 2009; Bernback et al., 1990; Bernback et al., 1989) together with pancreatic secretions (rich in the natural biosurfactants), bile salts and phospholipids. Bile salts can displace interfacial compounds,

promoting binding of the complex HPL-colipase at the oil-water surface area and hence lipolysis (Gallier, Acton, Garg, & Singh, 2016; Sarkar, Ye, & Singh, 2016; Salentinig, Phan, Hawley, & Boyd, 2015; Golding et al., 2011). Furthermore, the mixed micelles formed by bile salts and phospholipids are crucial for solubilization and absorption of products of digestion as well as many dietary hydrophobes, such as vitamins, cholesterol and sterols (Salentinig et al., 2015; Golding & Wooster, 2010). With the continuous arrival of chyme and the release of hydrolysis products in the intestinal phase, interactions between such molecules and emulsion droplets appear to be complex and are little understood (Singh et al., 2009).

In vitro studies have contributed to clarify some points about the influence of the quantity and quality of the interface on the digestion process (Gallier et al., 2016; Bourlieu & Michalski, 2015). Considering the quantity of interface (specific surface area), it was recently demonstrated to have a direct relation with the gastric lipolysis and proteolysis in bovine IF (comparison of model IF having precisely the same chemical composition but variable specific surfaces) (Bourlieu et al., 2015b). Conversely, in an *in vivo* study on the preterm newborn, Armand et al. (1996) reported higher gastric lipolysis levels in HM compared with IF (which is homogenized), with values around ~17% and 9% at 30 min, respectively. These results suggested a lower accessibility of HGL to the TG core in the droplet of IF compared to HM, which was justified by the differences on the quality of the interface. In this case, however, meal compositions were also different. Thus, although results considering the quality of the interface are conflicting, gastric lipase seems to be sensitive to the type of phospholipids present on the surface (Bourlieu et al., 2016) and to the absorption of proteins on the fat droplets interface, which could reduce the affinity of HGL to the substrate (Garcia et al., 2014).

Whether this is also applicable for HM, with its higher fat and lower protein content compared to bovine milk and IF, remains to be elucidated.

1.4.6. Studying the infant digestion

Studying digestion requires the use of *in vitro* or *in vivo* models. The choice between these two modalities depends on many factors such as ethical, technical and financial considerations.

In vivo digestion: animal and human models

Due to ethical and practical reasons, few *in vivo* studies dealing with infant digestion are available in the literature, most of them being published up to the 1990s. Usually they concern preterm, who are more often hospitalized than term infants, and are frequently fed through a nasogastric tube, which can be used for the collection of gastric contents by aspiration. Studies concerning the intestinal phase of the digestion are rarer due to the access difficulty and necessity of invasive approaches (Bourlieu et al., 2014).

To overcome these problems, the use of animals can be an alternative. The two most frequently animal as a model of human adult digestion used in literature for digestion studies are rat and pig (Ménard & Dupont, 2014; Gallier et al., 2013b; Gallier et al., 2013a; Deglaire & Moughan, 2012). The pig constitutes the preferred animal model for nutrition researchers' due to its digestive organization and eating habits, both similar to that of human beings (Deglaire & Moughan, 2012; Deglaire, Bos, Tome, & Moughan, 2009). Considering infant digestion, the piglet has been used as a model for studying digestive physiology and kinetics of hydrolysis (Le Hueron-Luron et al., 2016; Bouzerzour et al., 2012; Darragh & Moughan, 1995; Moughan, Birtles, Cranwell, Smith, & Pedraza, 1992). In rodents, however, their organs at birth are too immature and far from representing human development during fetal and neonatal period (Calder et al., 2006).

Digestion of nutrients can be studied by evaluating their kinetics of hydrolysis, studied by evaluating their bioavailability (proportion of digested and absorbed nutrients reaching the bloodstream) by taking blood samples at specific times before and after food consumption (Bornhorst, Gouseti, Wickham, & Bakalis, 2016). The most frequent nutrients evaluated are plasmatic AA, TG and glucose. The major difficulty in these studies is differentiating exogenous (dietary) and endogenous nutrients, which can be solved by adding a marker to the food (stable isotope) (Deglaire et al., 2008). Besides blood sampling, digesta can also be sampled in order to evaluate their digestibility, an estimation of bioavailability. This measure is indirect, as the non-digested quantity of nutrients is measured.

In vivo studies using animals or humans usually provide the most accurate results, but they are time consuming, costly and difficult to implement in terms of ethical and technical limitations. Moreover, due to high inter-individual variability large sample is needed (Bornhorst et al., 2016). In order to face this difficulty, much effort has been devoted to the development of pertinent *in vitro* models.

In vitro models simulating infant digestion

In comparison to *in vivo* models, *in vitro* digestion models are cost reduction, time saving, have better repeatability and reproducibility and limitation of ethical constraints (Ménard & Dupont, 2014).

Even with various limitations, *in vitro* models are useful for studying food kinetics of digestive hydrolysis and structural changes under simulated gastrointestinal digestion. They allow the screening of various foods by evaluating the bioaccessibility (proportion of nutrients that are digested and then available for absorption) before conducting *in vivo* trials in animals or in humans on a limited number of food matrices. Models can be simple or sophisticated and expensive, but the study of absorption by *in vitro* models is still limited. Other important limit is that such models

cannot mimic the hormonal feed-back mechanisms and neural interactions (Guerra et al., 2012; Wickham, Faulks, & Mills, 2009).

In vitro digestion studies can be static (Wada & Lonnerdal, 2015a; Lueamsaisuk, Lentle, MacGibbon, Matia-Merino, & Golding, 2014; Wada & Lonnerdal, 2014; Dupont et al., 2010b), semi-dynamic (Bourlieu et al., 2015b; Amara et al., 2014) or dynamic (Ménard et al., 2014; Shani-Levi, Levi-Tal, & Lesmes, 2013; Blanquet et al., 2004). Most models omit the oral phase, especially for those studying protein and lipid digestion.

Static models are widely used as they are quite easy to set up and do not require specific tools. A single set of parameters is set up at the start of the digestion (pH, meal dilution, enzyme concentration, mixing), without any intervention during the digestion time (no pH adjustment, no flow of enzymes and no emptying). These systems are used for the study of digestion of purified molecules (*i.e.* to assess the potential allergenicity of proteins) but also for screening different food matrices, when a choice has to be made among a large number of formulations before studying digestion on a limited number of matrices on more complex systems (Ménard & Dupont, 2014).

In the semi-dynamic model, a pH-stat device can be programmed to establish subsequent stage of pH, which is adjusted by the addition of simulated gastric fluid, resulting in variation of the pH, enzymes and the dilution of the meal by gastric secretions. In this model, the FA released can be directly titrated by NaOH; however, there is no emptying and no flux among the compartments (Bourlieu et al., 2015b).

Since static and semi-dynamic models are overly simplified in their representation of the digestive tract, the direct applicability of findings to de facto digestive processes is limited. Recently developed, gastrointestinal dynamic models have incorporated a more realistic representation of the digestive tract, since they simulate the dynamic changes of pH, enzymatic output and activity, emptying rate, and flux between gastric and intestinal phases. These dynamic models allow the collection of samples from different compartments over the digestion process, providing data in terms of kinetics of disintegration and hydrolysis. Different dynamic models have been developed to mimic one or several compartments, but they are still commercially unavailable or very expensive (Ménard et al., 2014; Zhang et al., 2014; Kong & Singh, 2010; Minekus et al., 1999; Minekus, Marteau, Havenaar, & Huisintveld, 1995; Molly, Woestyne, & Verstraete, 1993).

The most popular gastrointestinal dynamic model is the TIM-1 (TNO gastrointestinal model 1), which includes stomach and the three parts of the small intestine (duodenum, jejunum, ileum) (Minekus et al., 1995). Controlled by a computer, this model takes into account parameters such as human temperature, gastric pH decrease, digestive secretions, emptying rates, peristalsis movements and nutrient absorption in the intestine by a dialysis system (Guerra et al., 2012). Blanquet et al. (2004)

applied the TIM-1 model to study the behavior of oral drug dosage under *in vitro* infant digestion. More recently, a TIM-2 model was developed from TIM1 and additionally can mimic the microbiota (Yoo & Chen, 2006).

A more simplified gastrointestinal dynamic system, DIDGI® (INRA, France) contains two successive compartments simulating the stomach and the small intestine, and is controlled by the STORM® software. It simulates the flows of ingested food and among compartments, digestive secretions, the gastric pH decrease, emptying rates, temperature (Ménard et al., 2014). However, this model does not mimic absorption and the continuous mixing applied in order to avoid phase separation does not simulate peristaltic movements, which could artificially modulate the structures formed during digestion. This model was validated to simulate infant digestion on piglets.

An approach combining *in vitro* models and human intestinal cells in culture has been proposed, integrating active and facilitated transport processes and brush border enzyme activities (Poquet & Wooster, 2016; Abrahamse et al., 2012). Notably, Caco-2 cells have been used to investigate the intestinal absorption and lipid metabolism of lipolysis products in the intestinal epithelium (Lipkie et al., 2014; Vors et al., 2012; Andersson, Hernell, Blackberg, Falt, & Lindquist, 2011).

Set up of in vitro models

Developing relevant *in vitro* models of digestion relies on the availability of *in vivo* data about the digestive conditions in the course of the meal digestion. Anatomical and physiological parameters determining these conditions depend on age, meal, fed conditions and other specific characteristics from the target to simulate (*e.g.* preterm *versus* term newborns) (Bourlieu et al., 2014). Previous reviews have compiled the physiologic data available in literature about infant digestive conditions (Poquet & Wooster, 2016; Bourlieu et al., 2014; Abrahamse et al., 2012). Notably, Bourlieu et al. (2014) underlined the immaturity in the digestive conditions in terms of enzymatic (type of enzymes and level of activity) and non-enzymatic (milk-based diet, frequency of feeding, bile salts concentrations) parameters.

The parameters used on *in vitro* models for digestion are different among studies, which makes comparison between data delicate. For instance, the enzymatic parameters are scarcely expressed in international units (*i.e.* internal units/mL of gastro or intestinal content) and the enzyme characterization and activity are most of the time omitted in the digestion protocol description. Furthermore, duration of digestion and the meal proportion in the total volume widely vary among the studies (Minekus et al., 2014). Regarding infant models, most of the studies give no clear definition of the gestational or postnatal age (Shani-Levi et al., 2017). To face this challenge, the INFOGEST network was created for bringing together the scientific community in the field of

digestion. This group recently developed a consensus protocol for mimicking digestion at the adult stage with an *in vitro* static model (Minekus et al., 2014). However, there is no physiologically pertinent harmonized model of *in vitro* static or dynamic digestion at the infant stage.

Not only the level and flow of enzymes and bile salts consist key parameters for simulating physiological conditions during digestion, but also their type and origin. The commercially available enzymes and bile salts that are closer to the functionality of human digestive enzymes are often of porcine or bovine origin. The enzymes most commonly added in the digestion *in vitro* systems are pepsin, chymotrypsin, trypsin, lipase and amylase (Nguyen, Bhandari, Cichero, & Prakash, 2015; Ménard & Dupont, 2014). Few studies used human enzymes after collecting gastric or intestinal juices in adults (Almaas et al., 2006) or infants (Chatterton et al., 2004).

In the gastric phase, the low cost of porcine pepsin and its high homology with human pepsin (84%) support a regular use of porcine pepsin in *in vitro* digestion models (Minekus et al., 2014). The choice of the type of gastric lipase is a major concern for *in vitro* digestion models, besides concerns about the enzyme concentration, activity or definition of lipase unites (U). Since gastric lipase of animal origin is not commercially available, most of *in vitro* gastric digestion models use no lipase or microbial lipase, without evaluation of their correspondence with HGL. In fact, HGL presents some specific and unique properties which determine lipolysis in terms of amounts and type of partial glycerides and free FA released (*i.e. sn*-3 stereospecificity for TG hydrolysis, stability and activity in acid conditions, resistance to pepsin and high tensioactivity allowing penetration in phospholipids layers and resistance to bile salts) (Sams et al., 2016). Furthermore, the activity of microbial lipases is reported to be high also at neutral pH, thus having a larger contribution to lipolysis in the small intestine as compared to HGL (Abrahamse et al., 2012). Approaches to obtain sufficient quantities of gastric lipase have been reported. For instance, rabbit gastric lipase has been proposed as a pertinent replacement for HGL in *in vitro* models simulating gastric digestion (Sams et al., 2016; Moreau, Gargouri, Lecat, Junien, & Verger, 1988). However, this lipase is difficult to obtain commercially.

Concerning the simulated small intestinal digestion, the most important components are the pancreatic enzymes and the bile. Using individual enzymes is possible but expensive; thus, a suitable option is the use of porcine pancreatin, which contains the most important human pancreatic enzymes, *i.e.* trypsin, lipase and amylase (Nguyen et al., 2015; Minekus et al., 2014). However, there is a significant variation in pancreatin from supplier to supplier, and even batch to batch (Lohr et al., 2009). The activity of these enzymes should be determined before the use of porcine pancreatin, and then the amount added should be based on the tryptic or lipolytic activity, depending on the objective of the study. In the case of bile extract, usually from porcine or bovine origin, the concentration of bile salts need to be determined (Minekus et al., 2014).

Chapter 2 – Gastrointestinal dynamic *in vitro* digestion of human milk in term and preterm infants: impact of Holder pasteurization

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Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under *in vitro* dynamic term newborn digestion

Samira C. de Oliveira [a,b], Amélie Deglaire [a,b], Olivia Ménard [a,b], Amandine Bellanger [c], Florence Rousseau [a,b], Gwénaële Henry [a,b], Emelyne Dirson [c], Frédéric Carrière [d], Didier Dupont [a,b], Claire Bourlieu [a,b,*]

[a] INRA, UMR 1253, Science et Technologie du Lait et de l'Oeuf, 65 Rue de St Brieuc, 35042 Rennes, France
[b] Agrocampus Ouest, UMR 1253, Science et Technologie du Lait et de l'Oeuf, 65 Rue de St Brieuc, 35042 Rennes, France
[c] Centre Hospitalier Universitaire de Rennes, Département de Pédiatrie, 16 Boulevard de Bulgarie, 35203 Rennes, France
[d] CNRS, Aix Marseille Université, UMR 7282 Enzymologie Interfaciale & Physiologie de la Lipolyse, 31 Chemin Joseph Aiguier, 13402 Marseille, France

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Impact of pasteurization of human milk on preterm newborn *in vitro* digestion: Gastrointestinal disintegration, lipolysis and proteolysis

Samira C. de Oliveira [a], Claire Bourlieu [a], Olivia Ménard [a], Amandine Bellanger [b], Gwénaële Henry [a], Florence Rousseau [a], Emelyne Dirson [b], Frédéric Carrière [c], Didier Dupont [a], Amélie Deglaire [a,*]

[a] STLO, Agrocampus Ouest, INRA, Rennes, France
[b] Centre Hospitalier Universitaire de Rennes, Département de Pédiatrie, Rennes, France
[c] CNRS, Aix Marseille Université, UMR7282 Enzymologie Interfaciale & Physiologie de la Lipolyse, Marseille, France

HM is recognized as the best option for nourishing both term and preterm infants. When breastfeeding is not possible, Holder-pasteurized HM is often provided by HMB for hospitalized vulnerable infants. PHM from HMB is in first instance given to preterm infants, but term infants are also potential recipients, mainly in the presence of gastrointestinal disorders. As discussed in the bibliographic review (chapter 1, section 1.3), Holder pasteurization is recognized for modifying the composition, the structure and the bioactivity of HM, notably by inactivating endogenous enzymes such as BSSL. Nevertheless, it remains unclear how these modifications affect the digestion of HM. In this context, the aim of this chapter was to answer the following question:

Does Holder pasteurization affect the gastrointestinal digestion HM in term and preterm infants as simulated in vitro?

In order to answer this question, we used an original *in vitro* dynamic system developed by our research group (DIDGI®, INRA, France), which was validated to simulate infant digestion on piglets (Ménard et al., 2014). As the physiologic digestive conditions vary with the infant maturity and especially between term and preterm infants, we have proposed different parameters to simulate the gastrointestinal digestion at each of these stages, considering infants at the postnatal age of four weeks. Pools of either term or preterm HM (adapted to each respective stage) from five anonymous donors were digested as raw or pasteurized. Samples were withdrawn at different times during digestion and analyzed in order to assess the kinetics of lipolysis, proteolysis and emulsion disintegration of the different milks.

Corresponding published articles concerning term HM digested by term infants and preterm HM digested by preterm infants are presented in sections 2.1 and 2.2, respectively. A brief complementary discussion comparing the impact of Holder pasteurization in term and preterm stages is presented in section 2.3.

2.1. Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under *in vitro* dynamic term newborn digestion

2.1.1. Abstract

When the mother's own human milk is unavailable or limited, pasteurized human milk from milk banks is preferentially administered instead of infant formula, especially for vulnerable hospitalized neonates. Holder pasteurization (62.5 °C, 30 min) may alter human milk composition and structure, which may modulate its digestive behavior. An *in vitro* dynamic system was set up to simulate the gastrointestinal digestion of term newborns in order to compare the kinetics of lipolysis, proteolysis and structural disintegration of raw versus pasteurized human milk. Human milk from 5 donors was pooled. Half of the pool was either administrated raw (RHM) or pasteurized (PHM). Digestions were conducted at least in duplicate for RHM and PHM. Heat-induced protein aggregation was observed in PHM. During gastric digestion, β-casein was proteolyzed significantly faster for PHM than for RHM ($p < 0.05$), whereas lactoferrin tended to be proteolyzed slower ($p = 0.07$) for PHM. Pasteurization selectively affected the intestinal release of some amino acids. At any time of the gastrointestinal digestion, the lipolysis of PHM was significantly lower than that of RHM, but no impact was observed on the profile of released fatty acids. RHM presented a structural destabilization after 60 min of gastric digestion, while there was no large variation for PHM. In the intestinal phase, the evolution of the particle sizes was rather similar. Overall, Holder pasteurization impacted the proteolysis, lipolysis and disintegration of human milk. However, this impact was limited and the physiologic and metabolic consequences remain to be investigated.

Keywords: human milk; *in vitro* dynamic digestion; lipolysis; proteolysis; Holder pasteurization; term newborn.

2.1.2. Introduction

Human milk is the ideal food for infant nutrition, allowing optimal growth and providing several short and long-term health benefits (Le Huerou-Luron, Blat, & Boudry, 2010; Horta, Bahl, Martines, & Victora, 2007). Numerous human milk components, or their digestive products, are known to play beneficial functions such as the improvement of digestive maturity, the regulation of microbiota development and of the immune system (Donovan, 2006; Labbok et al., 2004; Goldman, 2000). When the mother's own human milk is unavailable or limited, pasteurized human milk from milk banks is preferentially administered instead of infant formulas, especially for vulnerable hospitalized neonates (Wight, 2001). In order to meet the infant nutrient requirements and also to respect the digestive

immaturity of the neonates, this practice is more and more encouraged by public health policies and is officially recommended (Arslanoglu et al., 2013; American Academy of Pediatrics, 2012). Several countries (*e.g.* France, Germany, Australia, Brazil) have developed their own guidelines to implement and regulate human milk banks (Vieira et al., 2011; Arnold, 2006). These regulations concern all the steps of handling, processing and storing of human milk, in order to assure its microbiological safety and nutritional quality. For sanitary reasons, the Holder pasteurization (62.5 °C, 30 min) of human milk is imposed by the worldwide guidelines. In addition, two freeze-thaw cycles are applied during milk storage: first from milk expression to pasteurization and last from pasteurization to administration (Borgo et al., 2014). These physical treatments (pasteurization and freeze–thaw) may partially modify the composition and the structure of the human milk, which could modulate its digestive behavior.

The consequences of pasteurization on the human milk composition have been explored previously, though some points remain unclear. Heating the human milk degrades some immunological and nutritional components such as immunoglobulins (IgA, IgG and IgM) (Koenig, de Albuquerque Diniz, Barbosa, & Vaz, 2005), vitamins (folacin, vitamins C and B6) (Molto-Puigmarti, Permanyer, Castellote, & Lopez-Sabater, 2011; Vanzoerengrobben, Schrijver, Vandenberg, & Berger, 1987) and enzymes (lysozyme, lactoperoxidase, lipoprotein lipase, bile salt stimulated lipase) (Akinbi et al., 2010; Henderson et al., 1998). Besides these alterations, heat treatments together with freeze–thaw cycles of human milk can induce the disruption of the milk fat globules (Vieira et al., 2011; Wardell et al., 1981), protein denaturation and some aggregation of proteins on the fat globule membrane (Raikos, 2010; Ye et al., 2004).

In terms of physiological and nutritional impacts, pasteurization of human milk may decrease lipid absorption (Andersson et al., 2007; Thomaz et al., 1999; Williamson et al., 1978) and may reduce the mean weight gain in neonates (Williamson et al., 1978). This may be explained by two main differences. First of all, the two endogenous lipases of human milk that facilitate lipids hydrolysis, lipoprotein lipase and bile salt stimulated lipase (BSSL), are completely inactivated during pasteurization (Henderson et al., 1998; Blackberg & Hernell, 1981). Secondly, some studies reported important losses of lipids and proteins during the nasogastric tube delivery of pasteurized human milk, which were attributed to increased adherence of the disrupted milk fat globules to flask and tube walls (Vieira et al., 2011; Stocks, Davies, Allen, & Sewell, 1985). Therefore, these two aspects may impact the energy and nutritional supply, and the ability of infant fed pasteurized human milk to digest lipids (Lindquist & Hernell, 2010; Armand et al., 1996).

Though the impact of bovine milk emulsion structure on its digestive behavior has been described *in vitro* (Bourlieu et al., 2015b; Golding et al., 2011; Macierzanka et al., 2009; Singh et al., 2009) and *in vivo* (Golding et al., 2011; Armand et al., 1999), it remains unclear how the biochemical and structural

consequences of Holder pasteurization impacts the digestion of the human milk. This understanding could represent a key step to optimize infant nutrition.

Despite its importance, several questions about infant digestion remain unsolved due to ethical and financial constraints of *in vivo* studies. In order to solve this problem, *in vitro* dynamic models are relevant tools which take into account the complexity of the physiological digestion and can mimic more realistically the digestive tract conditions and transient states of *in vivo* infant digestion (Ménard et al., 2014; Zhang et al., 2014; Shani-Levi et al., 2013). Moreover, an exhaustive compilation of *in vivo* digestion data from the literature (Bourlieu et al., 2014) has allowed a pertinent adjustment of parameters of *in vitro* digestion systems.

The objective of this study was twofold: i) to compare the digestive behavior of raw versus pasteurized human milk having the same macronutrients composition; ii) to set up an *in vitro* dynamic model that simulates the gastrointestinal conditions of a term newborn (gestational age >38 weeks). More specifically, this study aimed to determine the impact of Holder pasteurization on the kinetics of lipolysis, proteolysis and structural disintegration of human milk in the digestive tract of term newborns.

2.1.3. Materials and methods

Chemicals

Unless stated otherwise, chemicals were from commercial origin (Sigma-Aldrich, Saint-Quentin Fallavier, France).

Human milk samples

Mature human milk samples were obtained from a donor milk bank at the University Hospital Center in Rennes (France). Ethical approval for the study was granted by the Hospitals Ethics Committee (no. 13-12) and donors gave their informed written consent. Five lactating women collected their milk on average 11 weeks after term delivery (range: 6 to 14 weeks), following the recommendations of the milk bank. On average (± SD), donors were 32 ±2 years old and gestational age was 39 ± 1.5 weeks. Milk was stored at −20 °C after collection.

Human milk was thawed in a 4 °C temperature controlled room over 16 h. Equal volumes of milk from each donor were pooled and aliquoted in plastic bottles of 100 mL. Half of the pool, called raw human milk (RHM), went back to storage at −20 °C until digestion. The other half, called pasteurized human milk (PHM), underwent Holder pasteurization (62.5 °C, 30 min) before going back to storage at −20 °C. Conditions for pasteurization were the same than that performed in the milk bank, *i.e.* the same plastic bottles submerged in a water bath and the same temperature/time curve. Macronutrient composition

of raw and pasteurized human milk was assessed by infrared spectrophotometry, using a Human Milk Analyzer (Miris AB, Uppsala, Sweden) previously validated (Billard et al., 2015). Raw and pasteurized human milk had the same macronutrients composition, which consisted of 26.8 ± 1.5 g/L of fat, 10.7 ± 0.5 g/L of proteins and 79.8 ± 1.5 g/L of carbohydrates.

In vitro dynamic digestion model simulating term newborn conditions

Digestion conditions

Gastrointestinal digestions of RHM and PHM were performed in an *in vitro* dynamic system (DIDGI®, INRA, Paris, France) using a digestion model for term newborns. This digestion system has been previously validated against *in vivo* digestion of infant formula in piglets (Ménard et al., 2014). Parameters for gastric and intestinal phases were based on an exhaustive literature review of *in vivo* digestive conditions of the infant's upper gastrointestinal tract (Bourlieu et al., 2014). The parameters were chosen to closely mimic the digestive conditions of term newborns fed human milk at the postnatal age of four weeks (**Table 4**). The *in vitro* dynamic system was controlled by the STORM® software, which allows regulating and monitoring the digestive parameters (Guillemin, Perret, Picque, Ménard, & Cattenoz, 2010).

The pH acidification in the gastric compartment followed a linear regression obtained from *in vivo* data reported mainly for preterm newborns fed human milk or infant formula (Roman et al., 2007; Omari & Davidson, 2003; Mitchell, McClure, & Tubman, 2001; Armand et al., 1996; Smith, Kaminsky, & D'Souza, 1986; Sondheimer, Clark, & Gervaise, 1985; Cavell, 1983; Mason, 1962), since there is no abundant data from term newborns. The initial pH was set at the meal pH. Fasted conditions were based on 2 mL of simulated gastric fluid (SGF) adjusted at pH 2.7, as reported in fasted state for preterm newborn infants fed every 3 h (Roman et al., 2007; Omari & Davidson, 2003; Mitchell et al., 2001; Armand et al., 1996; Smith et al., 1986; Sondheimer et al., 1985). Concerning the intestinal compartment, the pH was constant and fixed at 6.2.

The transit time in the stomach and in the intestine followed an exponential pattern fitted by the mathematical model described by Elashoff, Reedy, and Meyer (1982). Gastric emptying of human milk was determined by fitting data from Billeaud, Guillet, and Sandler (1990), where 47 min corresponded to the estimated half-time of emptying (t1/2) of term newborns fed human milk.

Gastric lipase and pepsin amounts were derived from data obtained in preterm newborns (Roman et al., 2007; Armand et al., 1996), assuming that their activities increased with body weight (which is linked to the digestive maturity). The mean body weight of term newborns at four weeks of age was considered 4.25 kg (WHO, 2006). A freeze-dried rabbit gastric extract (RGE) was employed in the gastric phase, since rabbit gastric lipase is reported to be a relevant option for *in vitro* gastric digestion

studies, presenting 84% of homology with human gastric lipase (Capolino et al., 2011; Moreau et al., 1988). RGE was obtained after soaking rabbit stomachs in an appropriate buffer and contained lipase and pepsin. The added amount of RGE, which fully covered the required lipase activity, covered 97.3% of the pepsin activity required to simulate term newborn gastric digestion (**Table 4**). RGE was thus the unique source of pepsin. Bovine bile and porcine pancreatin extracts were added to reach the average values reported for term newborns in postprandial intestinal conditions: contents of the bile salts cholic acid and chenodeoxycholic acids for bile (Signer, Murphy, Edkins, & Anderson, 1974), and lipase activity for porcine pancreatin (Norman, Strandvik, & Ojamae, 1972). Following the lipase activity, the amounts of trypsin and chymotrypsin added were 148 and 21 U/mL, respectively. The protocols used to determine the bovine bile salts concentration and the enzymes activities have been described by Minekus et al. (2014) in the supplementarymaterial. Concerning the RGE, its lipase activity was measured under standard assay conditions of human gastric lipase (pH 5.5, 37 °C), using tributyrin as substrate (Gargouri et al., 1986).

Table 4. Gastrointestinal conditions for *in vitro* dynamic digestion of human milk simulating term conditions

Gastric conditions (37°C)		
SGF	Na^+	94 mmol/L
(stock solution adjusted at pH 6.5)	K^+	13 mmol/L
	Cl^-	122 mmol/L
Fasted state / initial conditions	SGF	2 mL
	pH	2.7
Milk ingested	Total volume	100 mL
	Flow rate	10 mL/min from 0 to 10 min
Gastric pH (acidification curve)	pH = -0.0155*t + milk pH	
	with *t*: time after ingestion in min	
SGF + enzymes (RGE)	Gastric lipase	19.2 U/mL
	Pepsin	268 U/mL
	Flow rate	1 mL/min from 0 to 10 min
		0.5 mL/min from 10 to 180 min
Gastric emptying	$t_{1/2}$	47 min
(Elashoff fitting)	β	0.9
Intestinal conditions (37°C)		
SIF	Na^+	164 mmol/L
(stock solution adjusted at pH 6.2)	K^+	10 mmol/L
	Ca^{2+}	119 mmol/L
Intestinal pH	6.2	
SIF + bile	Bile salts	3.1 mmol/L
	Flow rate	0.5 mL/min from 0 to 180 min
SIF + pancreatin	Pancreatic lipase	90 U/mL
	Flow rate	0.25 mL/min from 1 to 180 min
Intestinal emptying	$t_{1/2}$	200 min
(Elashoff fitting)	β	2.2

RGE, rabbit gastric extract; SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

Digestion experiment and sampling

Digestion experiments were performed over three hours, at least in duplicate for each matrix (n = 2 for RHM and n = 3 for PHM). Samples were collected from the human milk (initial time T0) and from both compartments at 30, 60, 90 and 120 min after the beginning of the digestion. An additional sample was aliquoted at 180 min from the intestinal compartment. Structural analysis by confocal microscopy and laser light scattering were performed immediately. Aliquots for lipid analysis were immediately submitted to lipid extraction, as detailed in section "lipid chemical analysis". Other aliquots were frozen at −20 °C directly (total fatty acids composition analysis) or after addition of the antiprotease phenylmethylsulfonyl fluoride (PMSF) at 0.37 g/L (SDS-PAGE and amino acid analyses).

SDS-PAGE

The electrophoretic analyses were performed using 4–12% polyacrylamide NuPAGE® Novex® Bis-Tris 15 well precast gels (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. All samples were diluted 4-fold with NuPAGE® LDS sample buffer and then treated with 0.5 M DL-dithiothreitol and distilled water. Mark 12 Unstained Standard (Invitrogen) was used as a molecular weight (Mw) marker, as reference of the position of the bands. RGE and porcine pancreatin were deposed on the gels as controls, corresponding to the concentrations present in the digestive fluids. Gels were fixed in 30% (*v/v*) ethanol, 10% (*v/v*) acetic acid and 60% (*v/v*) deionized water and were rinsed for 15 min in deionized water before staining with Coomassie Blue. Image analysis of SDS-PAGE gels was carried out using Image Scanner III (GE Healthcare Europe GbmH, Velizy-Villacoublay, France). After digitization of gels, the bands were selected and their gray intensity determined by densitometry using the software Image Quant TL™ (GE Healthcare Europe GbmH, Velizy-Villacoublay, France). Densitometry analyses of the SDS-PAGE gels were used for semi-quantification of protein levels. The percentage of each intact protein remaining in the gastric compartment at a given timewas estimated in comparison with the undigested human milk.

Amino acid analysis

The total amino acid (AA) contents were determined after acid hydrolysis of human milk, according to Davies and Thomas (1973). Hydrolysis reaction was done by adding 6N hydrochloric acid to 170 µL aliquot, at 110 °C for 24 h in vacuum sealed glass tubes. The sulfuric AA cysteine and methionine were measured as methionine sulphone and cysteic acid after performic acid oxidation. The free AA content was determined after deproteinization of the samples by sulfosalicylic acid (Merck-Eurolab, Grosseron S.A., Saint Herblain, France) according to the method of Mondino et al. (1972). Human milk and digesta aliquots (1 mL) were treated with 50 mg of sulfosalicylic acid, and incubated for 1 h at 4 °C. The

mixtures were centrifuged at 5000×g for 15 min at 4 °C and the supernatants were filtered through a 0.45 µm pore-size membrane (Sartorius, Palaiseau, France). The filtrate was diluted five times with a 0.2mol/L lithium citrate buffer (pH2.2) before injection. The AA analysis was then carried out by a cation exchange chromatography on a Biochrom30 automatic AA analyzer (Biochrom Ltd., Cambridge, G.B.) according to Moore, Spackman, and Stein (1958), using lithium citrate buffers as eluants and ninhydrin post-column reaction system. Tryptophan was not determined. The AA released during digestion was expressed as the percentage level of free AA versus total AA present in the undigested human milk. The level of free AA was expressed in g/100 g of human milk by dividing the AA content measured in the digesta by the proportion of human milk in this digesta. This value was determined thanks to the in and out volumes monitoring by the STORM® software.

Lipid chemical analysis

Total lipids (C8:0 to C24:0) were analyzed by gas chromatography coupled to a flame ionization detector (GC–FID) by direct transmethylation in the presence of internal standard (C13) without prior extraction, as described by Lopez-Lopez et al. (2002). The GC–FID conditions of analysis were similar to the one reported by Briard-Bion et al. (2008). Samples were injected in duplicate. The fatty acids (FA) methyl esters were identified by comparing the retention time with the standards and their relative contents were expressed as mass %.

The other lipids characterizations (lipid classes and profile in free fatty acids) were done after direct Folch extraction performed immediately after sample collection as described by Bourlieu et al. (2015b). The chloroform phase was recovered and stored at −20 °C until further analysis.

The lipid classes, *i.e.* residual triglycerides (TG), diglycerides (DG), monoglycerides (MG) and free fatty acids (FFA) in aliquots withdrawn from the digestions were analyzed using IATROSCAN MK5 equipment (Iatron Laboratories, Tokyo, Japan), which corresponds to thin-layer chromatography coupled to a FID (TLC–FID), as previously described (Carrière et al., 2005). The mass detection data were converted into moles by using the average molar masses (g/mol) calculated from the FA composition of human milk (TG: 832; DG: 585; MG: 339; FFA: 265). The instantaneous lipolysis degree during digestion was then expressed as the percentage level of FFA (in moles) versus the total acyl chains present in residual glycerides and FFA quantified at a given time, as the following equation:

$$LD = \frac{100*[FFA]}{(3*[TG] + 2*[DG] + [MG] + [FFA])} \quad (1)$$

with LD lipolysis degree in %, [FFA], [TG], [DG] and [MG] respectively the free fatty acids, triglycerides, diglycerides and monoglycerides molar concentration (mol/L).

FFA (C8:0 to C20:0) were analyzed after solid phase extraction by GC, using three internal standards (160 µL of C5, C11 and C17 at 0.5 mg/mL) added to aliquot prior solvent extraction, as previously described by Bourlieu et al. (2012). Samples were injected in triplicate. The relative contents of each FFA were expressed as mass %; or converted into moles and reported to the total acyl chains in human milk in order to calculate the lipolysis degree of the undigested human milk.

Structural characterization

The microstructure of human milk and digesta was observed using two apparatus, as previously described by Bourlieu et al. (2015b): i) a Nikon C1Si confocal laser scanning microscopy (CLSM) on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France), using three fluorescent dyes; ii) a Mastersizer 2000 (Malvern Instruments, Malvern, UK) laser light scattering with two laser sources. Particle size distributions were summarized by distribution curves.

Statistical analysis

The differences between groups during the 3 h postprandial period were tested by repeated-measures analysis of variance using the MIXED procedure with time as repeated factor under the SAS software (SAS 9.4; SAS Institute Inc., Cary, NC). For each parameter, eight different covariance structures for random statements [Unstructured, Compound Symmetry, Heterogeneous Compound Symmetry, Auto Regressive (1), Heterogeneous Auto Regressive (1), Auto Regressive Moving Average (1,1), Spatial Power and Toeplitz] were tested, and the most appropriate matrix was selected based on the fit statistics (AIC and BICC criterion). Post hoc tests were performed by using contrast analysis. A p-value b 0.05was considered as statistically significant. Results were expressed as means ± standard deviation (SD).

2.1.4. Results

Proteolysis kinetics during human milk digestion

Figure 13 shows the protein profiles of RHM and PHM during digestion. The main bands in Figure 13A correspond to lactoferrin, serum albumin, β-casein and α-lactalbumin (theoretical molecular weight without signal peptides of 76.30, 66.47, 23.86 and 14.08 kDa, respectively - UniProtKB/Swiss-Prot). The decrease observed in band intensity along the digestion was due to proteolysis but also to dilution by digestive secretions and emptying. As secretions output and emptying rate were the same for the two types of milk, the difference of intensities between RHM and PHM were thus interpreted as different proteolysis rates.

As observed in **Figure 13**A, the impact of pasteurization on the proteolysis kinetics differed according to the type of protein. Proteolysis of lactoferrin tended to be faster for PHM compared to RHM (p =

0.07, **Figure 13**C). In contrast, proteolysis of β-casein was significantly slower for PHM than for RHM ($p < 0.05$, **Figure 13**F). No significant difference was identified between RHM and PHM during the gastric digestion of serum albumin and α-lactalbumin (**Figure 13**D and E). Whatever the type of milk, after 120 min of gastric digestion, lactoferrin and β-casein were more extensively digested than serum albumin and α-lactalbumin (**Figure 13**C to F).

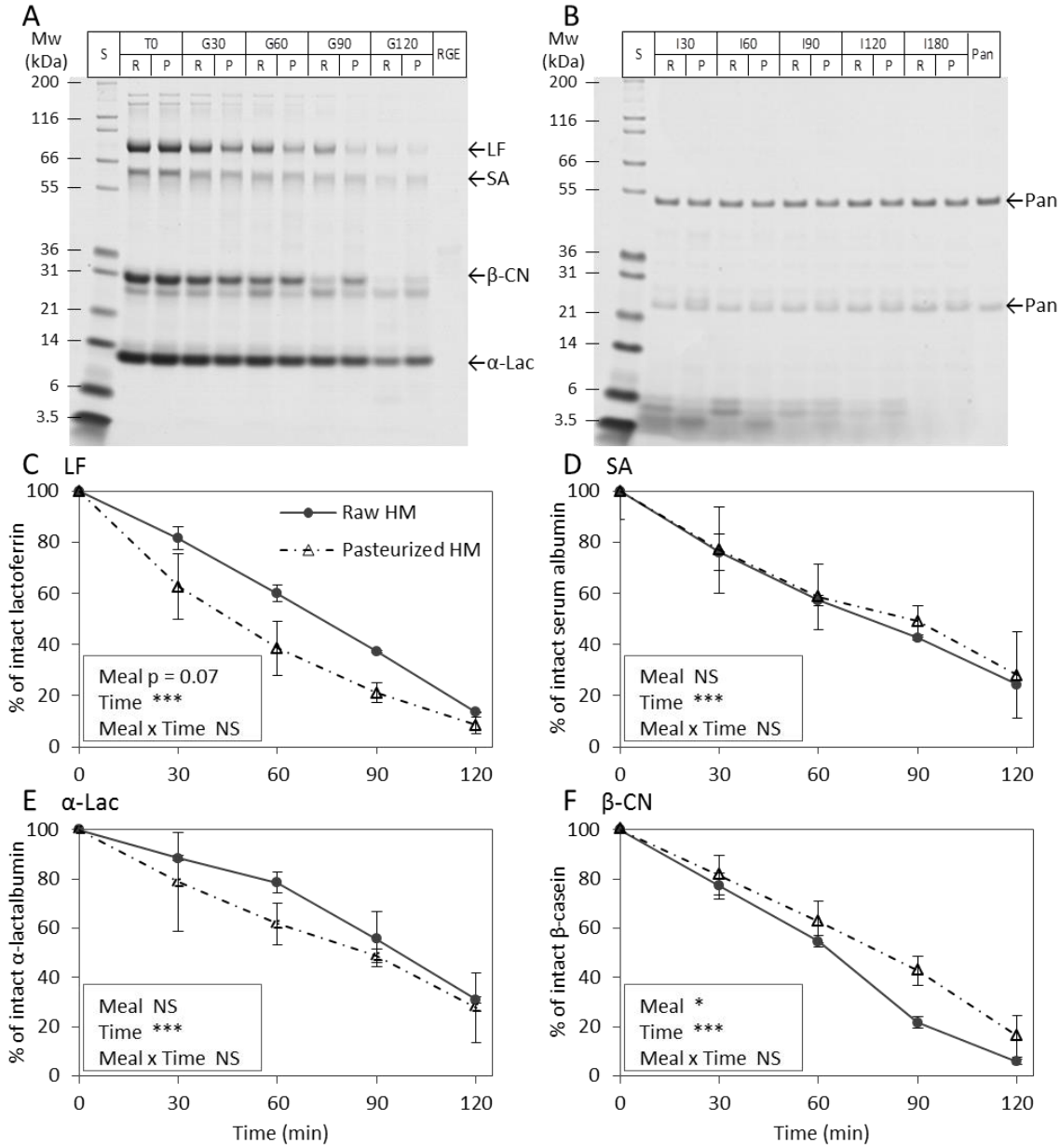


Figure 13. SDS-PAGE protein profiles of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion simulating term newborn conditions and the corresponding percentage of intact protein during gastric digestion as obtained by densitometry for lactoferrin (LF; panel C), serum albumin (SA; panel D), α-lactalbumin (α-Lac; panel E) and β-casein (β-CN; panel F). Raw (R) and pasteurized (P) human milk digesta appear side by side on the gels (A and B) for each time (left: R; right: P). Protein molecular mass standards (S) are on the left. Rabbit gastric extract (RGE) diluted in SGF and porcine pancreatin (Pan) diluted as in SIF are used as extra controls. Data point in panels C, D, E and F represents means ± SD (n = 2 for RHM and n = 3 for PHM). $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.1$ (NS). Data from undigested milk were not included in the statistical analysis.

Regarding the intestinal phase (**Figure 13**B), from 30 min onwards no intact protein from human milk was detected, suggesting that proteins were rapidly digested once arriving in the intestinal compartment. Only proteins from porcine pancreatin (Mw ~53 and 24 kDa) were present in the intestinal compartment at the same ratio throughout the digestion. Peptides arising from the protein digestion were visible in the lower part of the gel (Mw ~2 to 6 kDa). The peptide profiles largely differed between RHM and PHM at 30 and 60 min, with higher molecular weight peptides observed for RHM.

The AA contents of RHM and PHM before digestion were similar (**Table 5**). Some AA were present in the free form in RHM and PHM before digestion: in particular, 18.5% of the total glutamic acid and 3 – 5% of the total amount of cysteine, threonine, serine, glycine and alanine. The proportion of nitrogen coming from the total AA analyzed (α-amino nitrogen) before digestion represented 66% of total nitrogen. Considering the free AA released during gastric digestion, the amounts remained low and similar to the levels reported in the undigested RHM or PHM (data not shown).

Table 5. Amino acid composition of human milk before *in vitro* digestion

Amino acid *(g/ kg)*	Total Amino Acids	Free Amino Acids
Isoleucine	0.449	0.000
Leucine	0.877	0.005
Lysine	0.611	0.001
Methionine	0.181	0.000
Cysteine	0.186	0.007
Phenylalanine	0.364	0.000
Tyrosine	0.393	0.000
Threonine	0.393	0.010
Valine	0.470	0.004
Histidine	0.210	0.002
Glutamic acid	1.548	0.287
Aspartic acid	0.850	0.006
Proline	0.820	0.001
Serine	0.391	0.014
Glycine	0.201	0.008
Alanine	0.340	0.016
Arginine	0.297	0.000
Taurine	-	0.029
Amino nitrogen	1.090	0.041
Total nitrogen	1.650	

During the course of the intestinal digestion, the percentage of released AA increased significantly, as displayed in **Table 6**. Pasteurization affected the intestinal release of some AA, but the effect differed according to the AA. For methionine, proline and glycine, the percentage of released AA was significantly lower for PHM than for RHM during the entire course of the intestinal digestion, and only at 180 min of digestion for glutamic acid. On the contrary, for phenylalanine, tyrosine and isoleucine, the percentage of released AA was significantly higher for PHM than for RHM at some specific time points (**Table 6**).

Table 6. Amino acid released from RHM and PHM during intestinal *in vitro* dynamic digestion simulating term newborn conditions

Amino acid	Amino acid released during digestion (% w/w of total amino acids)										Effect of [1]		
	Initial milk		I60		I90		I120		I180		Meal	Time	Meal * Time
	RHM	PHM	RHM	PHM	RHM	PHM	RHM	PHM	RHM	PHM			
Isoleucine	0 ± 0	0 ± 0	8.4 ± 2.3	7.7 ± 0.5	12.2 ± 2.1	13.2 ± 1.5	16.5 ± 0.6[b]	18.7 ± 0.7[a]	23.6 ± 5.9	28.3 ± 0.9	NS	***	*
Leucine	0.5 ± 0.1	0.6 ± 0	25.2 ± 5.4	24.6 ± 1.2	30.2 ± 4.5	31.1 ± 1.6	33 ± 4.4	37.3 ± 1.9	47.7 ± 5.6	48.1 ± 2.4	NS	***	***
Lysine	0.2 ± 0.3	0.2 ± 0.1	40.4 ± 6.3	38.6 ± 1.9	50.2 ± 4.3	49.4 ± 2.6	56 ± 3.6	59 ± 2.7	82.7 ± 4.5	78.3 ± 2.7	NS	***	***
Methionine	0 ± 0	0 ± 0	19.6 ± 2.5	13.9 ± 0.7	27.0 ± 1.1	21.0 ± 1.5	33.0 ± 0.7	27.5 ± 1	46.7 ± 2.6	39.2 ± 2.2	*	***	NS
Cysteine	3.6 ± 0.1	3.7 ± 0	9 ± 1.7	8.4 ± 0.8	10.5 ± 1.1	10 ± 0.9	12 ± 0.6	12.3 ± 1.3	18.4 ± 2.6	19 ± 0.8	NS	***	**
Phenylalanine	0 ± 0	0 ± 0	26.4 ± 3.4	30.4 ± 1.2	35.2 ± 2.7[b]	40.4 ± 1.7[a]	40.1 ± 2.2[b]	49.7 ± 2.5[a]	58.4 ± 2.9[b]	63 ± 1.7[a]	NS	***	***
Tyrosine	0 ± 0	0 ± 0	49.7 ± 9.8	53.0 ± 2.4	58.3 ± 7.5	64.7 ± 3.2	61.2 ± 8.1[b]	75.2 ± 3.7[a]	89.6 ± 5.4	93.1 ± 3.8	NS	***	***
Threonine	2.6 ± 0.1	2.7 ± 0	14.9 ± 2.5	12.7 ± 0.6	17.5 ± 1.5	16.2 ± 0.9	21.5 ± 0.6	20.5 ± 1.1	31.7 ± 4.1	31.7 ± 1.2	NS	***	NS
Valine	0.8 ± 0.1	1 ± 0.3	12.5 ± 2.6	9.6 ± 0.5	15.3 ± 1.9	13.3 ± 0.7	17.6 ± 1	16.9 ± 0.9	25.6 ± 3.2	24.7 ± 1.1	NS	***	**
Histidine	0.9 ± 1.2	1.1 ± 1	10.7 ± 2.9	9.5 ± 0.8	14.9 ± 2.1	14 ± 1.9	20 ± 2.5	19.4 ± 3.4	28.8 ± 3.8	30.6 ± 3	NS	***	NS
Glutamic acid	18.6 ± 0.2	18.4 ± 0.4	27.9 ± 2.9	24.8 ± 1.6	30.6 ± 1.2	28.1 ± 1.5	31.8 ± 1.5	32.7 ± 1.3	46.8 ± 0.0[a]	42.7 ± 1.3[b]	NS	***	***
Aspartic acid	0.8 ± 0.1	0.8 ± 0.1	10.8 ± 1.6	10 ± 0.4	13.2 ± 0.5	12.4 ± 1.2	16.9 ± 2.1	15.8 ± 0.8	24.8 ± 2.5	25 ± 0.7	NS	***	NS
Proline	0.1 ± 0.1	0.1 ± 0	1 ± 0.2	0.7 ± 0	1.3 ± 0.1	1 ± 0.1	1.8 ± 0.1	1.3 ± 0	2.7 ± 0.4	2.3 ± 0.2	*	***	NS
Serine	3.5 ± 0.1	3.6 ± 0.1	17 ± 2.5	14 ± 0.6	18.9 ± 1.5	16.4 ± 0.8	21.1 ± 0.1	19.5 ± 0.6	31.1 ± 1.1	27.4 ± 2.1	NS	***	NS
Glycine	3.8 ± 0.4	4.1 ± 0.1	17.6 ± 1	16.4 ± 0.8	20.4 ± 0.3	19 ± 0.6	23.6 ± 2	22.4 ± 0.2	35.1 ± 0.1	33.2 ± 0.6	**	***	NS
Alanine	4.7 ± 0.3	4.9 ± 0.2	19 ± 2.5	18 ± 0.9	23 ± 1.4	22.7 ± 1.2	27.4 ± 0.4	28.2 ± 1.2	40.3 ± 3.5	41.3 ± 1.2	NS	***	NS
Arginine	0 ± 0	0 ± 0	65 ± 13.5	67.5 ± 3.9	74.1 ± 9.1	78.2 ± 4.8	77.8 ± 7.4	88.1 ± 4.6	113.6 ± 5.9	111.6 ± 3.3	NS	***	***
α-amino nitrogen[2]	3.4 ± 0.2	3.5 ± 0.1	24.1 ± 4.1	23.5 ± 1.1	28.6 ± 2.7	28.9 ± 1.5	31.8 ± 1.6	34.5 ± 1.5	46.6 ± 3.1	46.4 ± 1.2	NS	***	*

I, intestinal phase followed by time in min.

[1] Statistics were conducted on data from I60 to I180. Statistical significance: $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.1$ (NS).When there was a significant interaction, multiple comparisons of means were conducted. Data having a different letter within the same row and at the same time point significantly differed.

[2] Calculated from the proportion of α-amino nitrogen in the free compared to the total amino acids.

Lipolysis kinetics and fatty acid composition

The kinetics of lipolysis of RHM and PHM are shown in **Figure 14**. At any time of the gastrointestinal digestion, the lipolysis of PHM was significantly lower than that of RHM ($p < 0.05$). Before digestion, the milk pre-lipolysis was 5% lower in PHM than in RHM ($p < 0.05$). The lipolysis degree of both RHM and PHM sharply increased during the first 30 min of gastric digestion, and all over the intestinal phase. In contrast, the lipolysis did not evolve that much between 30 and 120 min of gastric digestion: in this compartment, lipolysis leveled off at 16.5 ± 5% for PHM and 24.1 ± 3.2% for RHM ($p < 0.05$) (**Figure 14**A). Higher levels were reached by the end of the intestinal digestion (180 min): 56.4 ± 3.8% for PHM *versus* 62.3 ± 6.0% for RHM (**Figure 14**B).

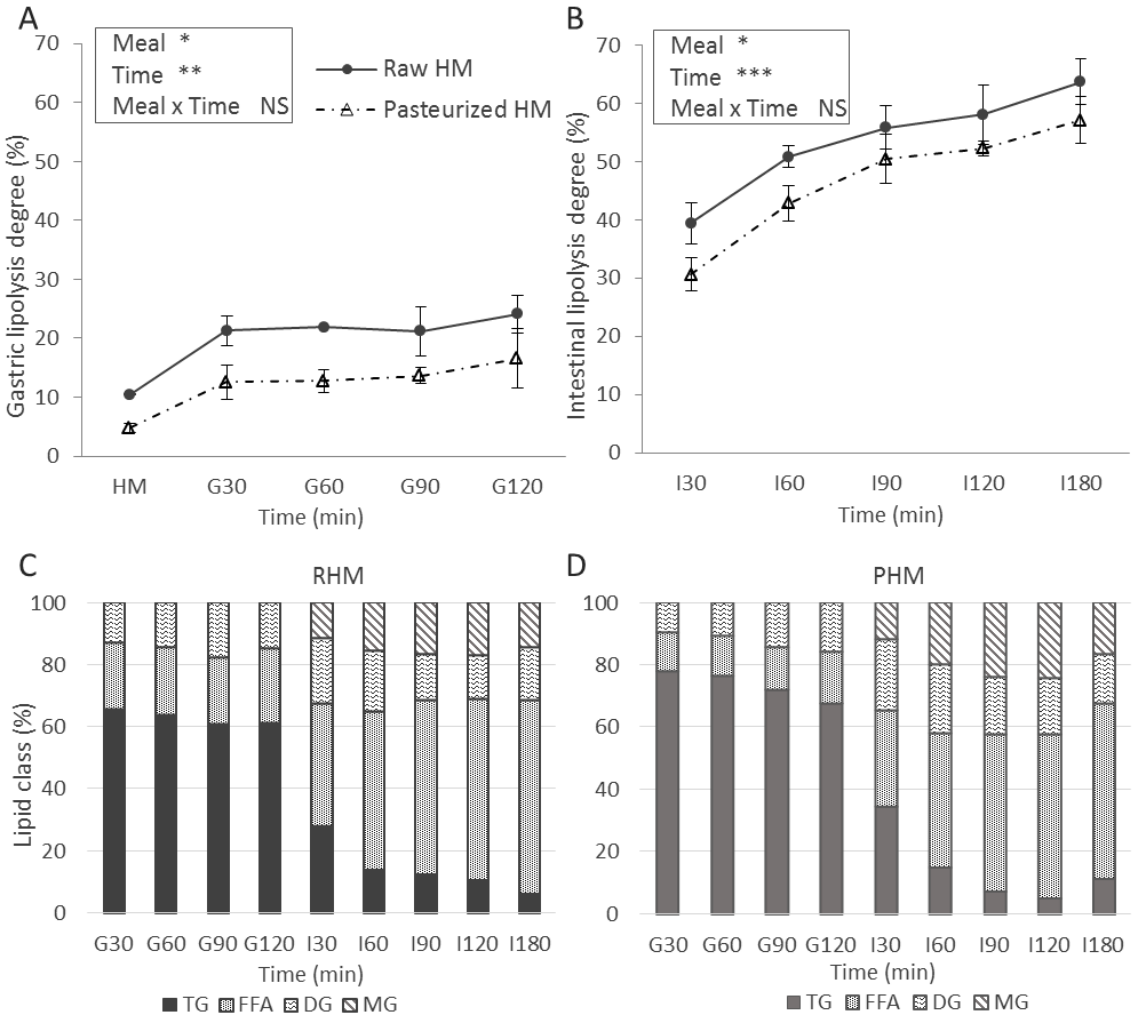


Figure 14. Kinetics of lipolysis of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion simulating newborn term conditions. The lipolysis degree was determined based on TLC–FID for digesta and on gas chromatography for undigested milk. Data point represents means (n = 2 for RHM and n = 3 for PHM) ± SD. $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.1$ (NS). The evolution of the lipid classes composition (acyl chains, mol%) of RHM (C) and PHM (D) during the gastrointestinal digestion was determined by TLC–FID. TG, FFA, DG and MG are respectively triglycerides, free fatty acids, diglycerides and monoglycerides.

The evolution of the acyl chains (mol%) from each lipid classes composition of RHM and PHM during the *in vitro* digestion was linked to the evolution of the lipolysis (**Figure 14**C and D). The lipid class composition over gastric phase was quite stable and was marked, as expected, by a higher percentage of TG in PHM, and by the early release, *i.e.* as soon as 30 min, of FFA and DG. The intestinal phase was marked by the progressive decrease of TG and an increase in the products of lipolysis, predominantly FFA along with DG and MG. MG was not detected in the gastric phase, but was observed since the beginning of the intestinal digestion of both RHM and PHM. MG levels were higher in PHM than in RHM from 60 to 120 min.

Figure 15 shows the acyl chains profile initially esterified in the matrices in comparison with the profile in released FA during the RHM (left panel) and PHM (right panel) *in vitro* digestions. No impact of pasteurization was observed on the profile of released FA. The pool of mature human milk used in this study contained 46.7% saturated FA and 53.3% unsaturated FA. The profile was dominated by five FA: oleic (C18:1 c9, 35.6%), palmitic (C16:0, 22.8%), linoleic (C18:2 c9, c12, 9.8%), stearic (C18:0, 7.5%) and myristic (C14:0, 6.3%) acid. The ratio ω-6/ω-3 was 9.2. During both gastric and intestinal phases, the major products of the lipolysis were C18:1 c9 and C16:0, followed by C18:0 and C18:2 c9, c12. During the lipolysis, whatever the type of meals, C18:1 c9 (30.1% to 49.9%) and C18:0 (9.8% to 18.6%) were selectively released as their relative amount was higher in the released FA fraction than in the total FA initially esterified. On the contrary, the proportion of released C16:0 (15.4 to 26.7%) tended to be lower when compared to the total FA initially esterified. During the course of gastric hydrolysis, the proportion of C18:1 c9 decreased relative to that of C16:0 and C18:0.

Structural changes during gastric and intestinal digestion

Concerning the size distribution of the particles, the average mode diameter measured in dissociating agent (sodium dodecyl sulfate, SDS) was higher for PHM (6.1 ± 0.1 µm) than for RHM (5.1 ± 0.2 µm). The specific surface areas developed by the fat globules were 5.0 ± 1.1 and 4.0 ± 0.0 m^2/g of lipid in RHM and PHM, respectively. In the same way, microscopic observations showed that pasteurization led to heat-induced protein aggregates in the soluble phase and at the interface of the human milk fat globule membrane (**Figure 16**). After 60 min of gastric digestion, there was a structural destabilization of RHM, with a bimodal distribution of the particle size (**Figure 17**A, mode 1 = 76.5 µm, mode 2 = 6.5 µm). The resulting aggregates were dissociated in the presence of SDS, quasi-restoring the initial particle size distribution (data not shown). On the contrary, there were no large variations of the particle size distribution between the start and the end of the gastric digestion of PHM: the average mode diameter was still of 6.5 µm at 120 min of gastric digestion (**Figure 17**B).

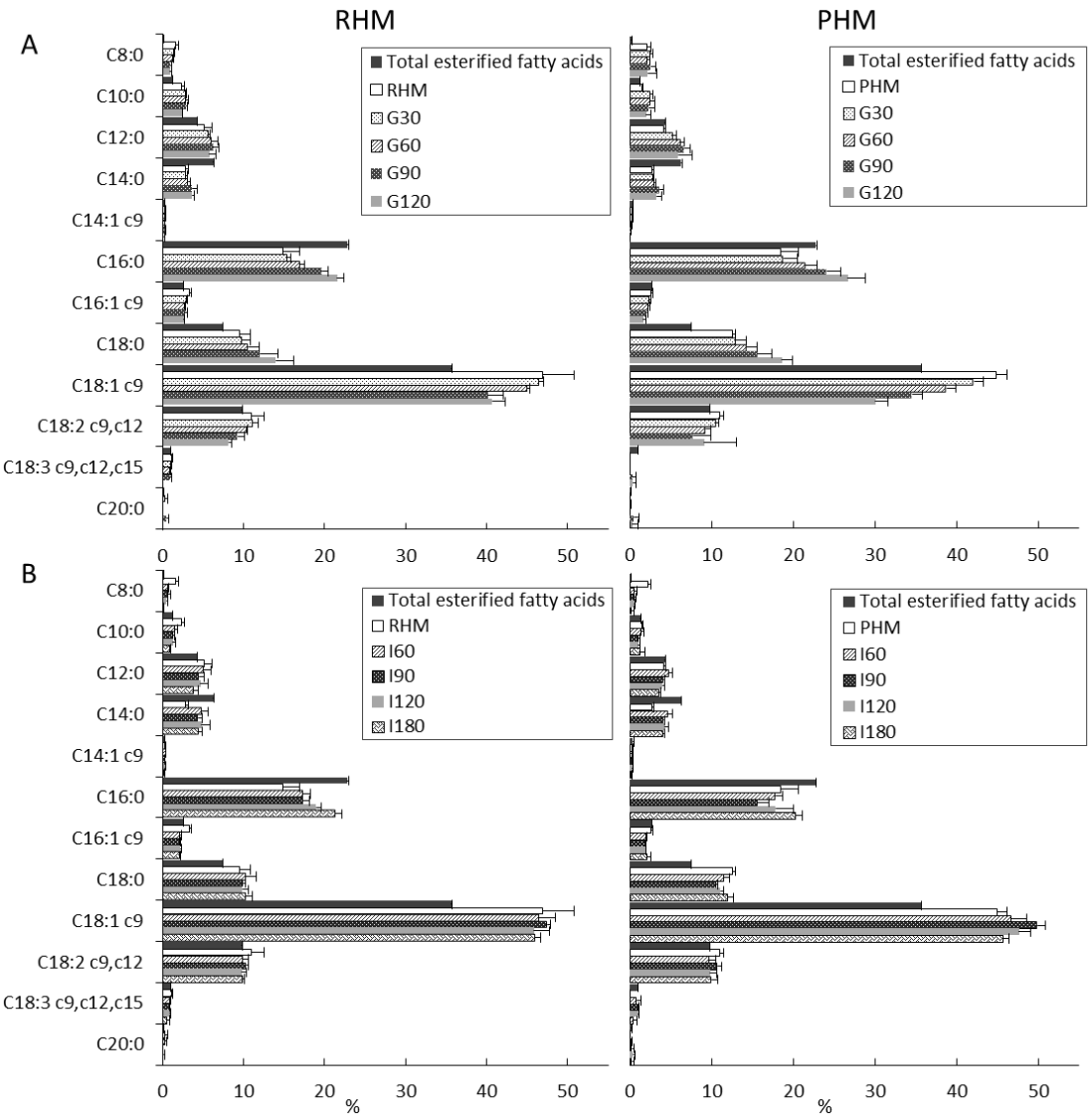


Figure 15. Acyl chains profiles initially esterified in human milk compared to free fatty acids released from RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion. Fatty acids were determined by gas chromatography. Data point represents means (n = 2 for RHM and n = 3 for PHM) ± SD.

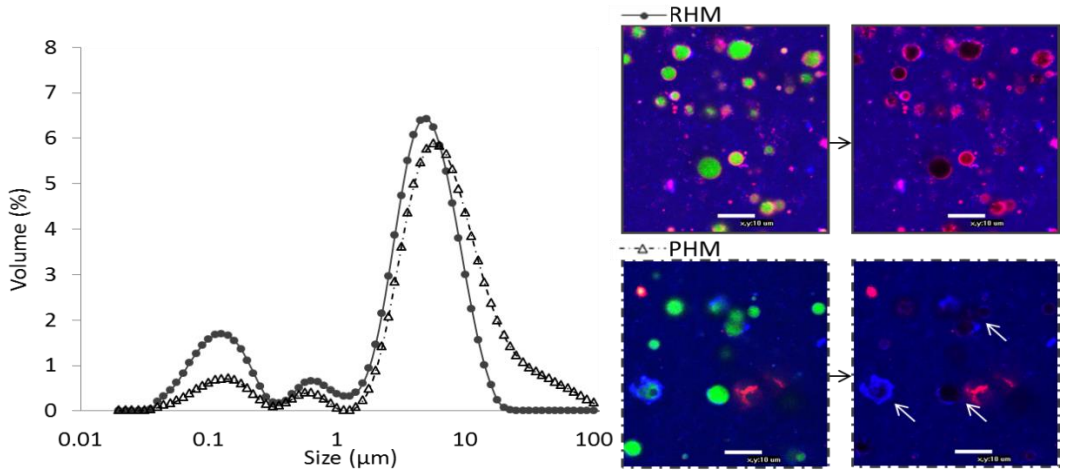


Figure 16. Particle size distributions (as determined by laser light scattering in SDS) and confocal laser scanning microscopy images (×60 zoom3) of undigested RHM and PHM. Data are mean of two digestions. Proteins are colored in blue (FastGreen®), apolar lipids in green (Lipidtox®) and polar lipids in red (Rhodamine-PE®). White arrows are pointing at heat-induced protein aggregates in PHM. Scale bars = 10 µm.

As observed by CLSM, the difference in initial structure was maintained during the gastric phase of digestion, which was dominated by protein aggregation observed from 30 min for both RHM and PHM (**Figure 18**A). From 60 up to 120 min the aggregates were smaller and more homogeneously distributed for PHM compared to RHM, which correspond to the destabilization observed by laser light scattering (**Figure 17**A). Less material was observed after prolonged digestion time as a result of hydrolysis, emptying and dilution by the gastric fluids. Interestingly, the milk fat globule structure persisted through the gastric digestion: the typical structure characterized by a hydrophobic core and enveloped by an amphiphilic membrane was still observed up to 120 min. At the end of the gastric digestion, these native fat globules were more frequently observed for PHM, whereas for RHM most of globules presented a mix of hydrophobic and amphiphilic compounds in their core. The rafts or micro-domains of the milk fat globule membrane were still visualized even below the protein aggregated layer (**Figure 17**C).

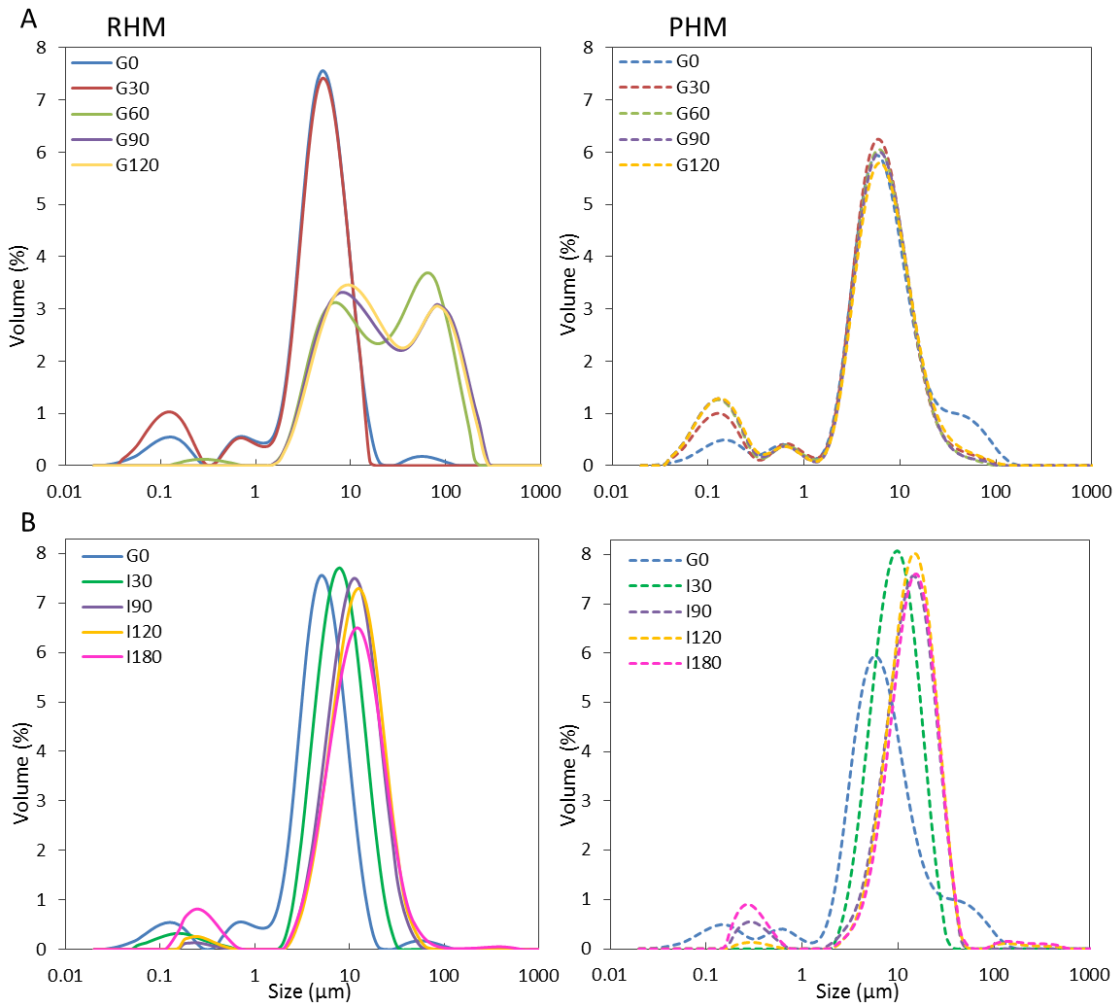
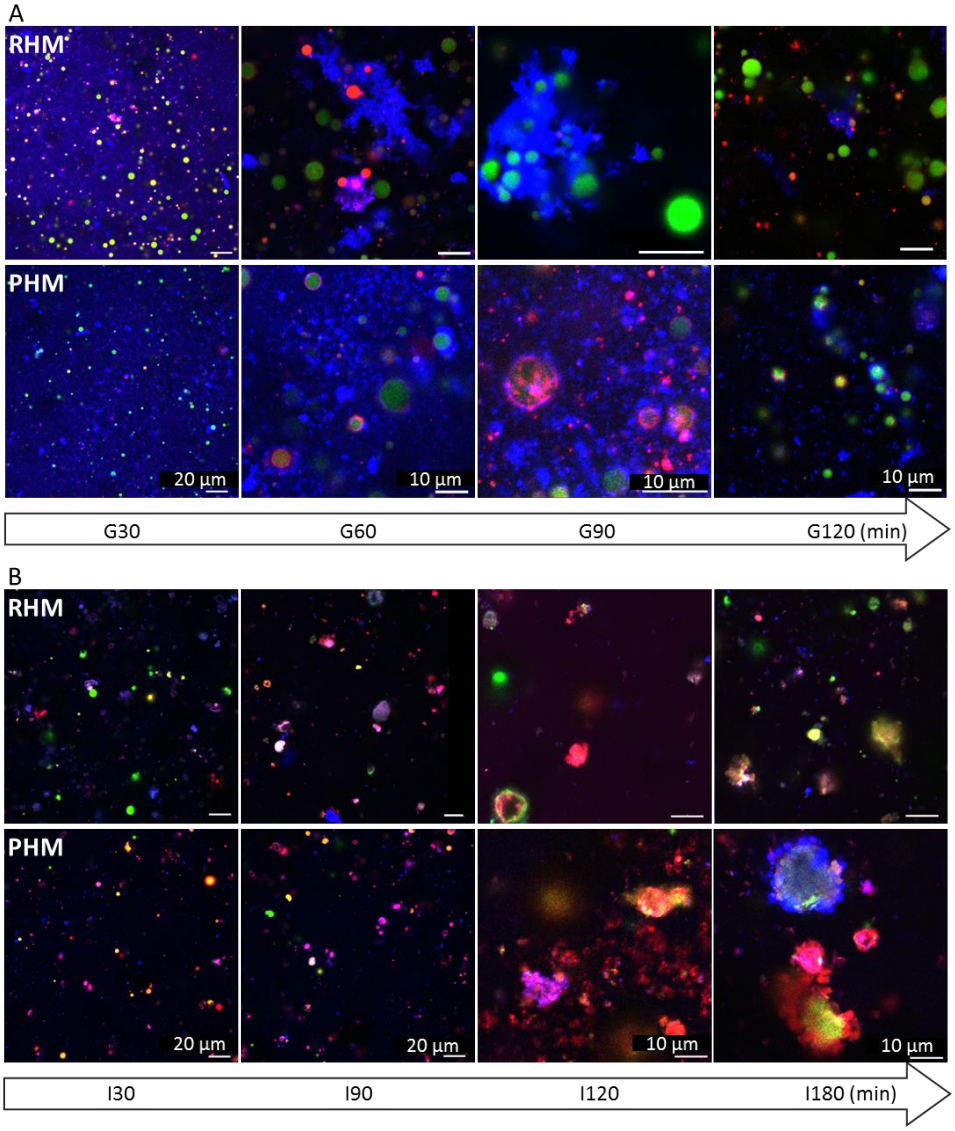


Figure 17. Particle size distributions of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion, as determined by laser light scattering in water. Data represents the average of two digestions, each of them being measured in triplicate.

In the intestinal phase, the evolution of the particle sizes was rather similar for RHM and PHM (**Figure 17**B), with an increase in the mode diameter between 30 and 180 min of intestinal digestion: from 8.1 ± 1.5 to 13.2 ± 1.0 µm for RHM, and from 10.3 ± 0.2 to 16.2 ± 1.0 µm for PHM. However, in PHM, bigger aggregates (from 150 to 290 µm) were observed at the end of the intestinal phase. CLSM indicated that the intestinal phase was marked by mixed aggregates of apolar and amphiphilic compounds, no proteins in the soluble phase and the quasi-disappearance of previously observed gastric protein aggregates (**Figure 18**B).



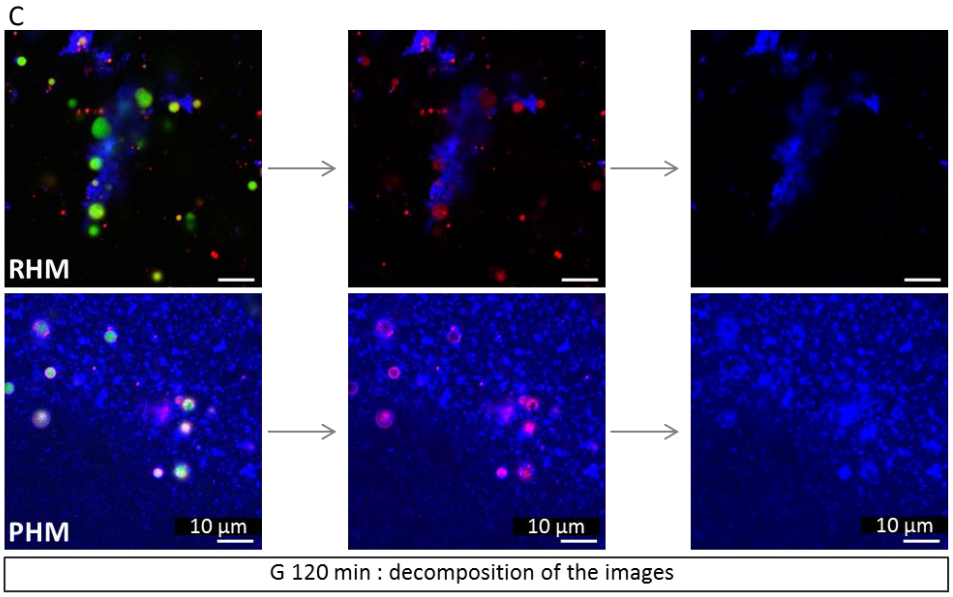


Figure 18. Confocal laser scanning microscopy images of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion. C) Decomposition of the images at 120 min of gastric digestion of RHM and PHM (photo 1: proteins, apolar and polar lipids; photo 2: proteins and polar lipids; photo 3: proteins). Each frame is the average of two observations (×60 zoom1 to 6). Proteins are colored in blue (FastGreen®), apolar lipids in green (Lipidtox®) and polar lipids in red (Rhodamine-PE®).

2.1.5. Discussion

This study has evidenced that Holder pasteurization affects the digestive disintegration and the kinetics of lipolysis and proteolysis of human milk in term newborns, as investigated using an *in vitro* dynamic gastrointestinal digestion model. Remarkably, it is the first time that the digestive behavior of raw *versus* pasteurized human milk having the same macronutrients composition is described. It is also the first time that heat-induced protein aggregation is observed after Holder pasteurization, *i.e.* a low temperature but long-time heat treatment. On the contrary, structural changes in cow's milk proteins after heat treatment with subsequent effects on the emulsion properties were previously described (Raikos, 2010). Heat-induced protein aggregation consists of the aggregation of whey proteins in the colloidal phase, which further form complexes with caseins on the surface of the casein micelle (Ye et al., 2004; Guyomarc'h, Law, & Dalgleish, 2003; Ye, Singh, Taylor, & Anema, 2002). From our data, we hypothesize that these structural changes may represent key elements to explain the impact of pasteurization on the gastric proteolysis of β-casein and lactoferrin.

Lactoferrin in human milk is in the holo conformation, which induces a closed molecule conformation and confers some resistance to proteolysis (Garcia-Montoya et al., 2012). Its globular structure is likely denatured by pasteurization (Kulmyrzaev, Levieux, & Dufour, 2005); once denatured and adsorbed at the surface of the micelles, lactoferrin may be more easily digested by proteolytic enzymes. In contrast, β-casein is sheltered inside the casein micelles and consequently less accessible to enzymes after whey protein aggregation. Indeed, other studies raised the hypothesis that heat treatments

applied to bovine milk increase the resistance of β-casein to digestion (Dupont et al., 2010b; Almaas et al., 2006). This modified susceptibility to proteolysis for human milk after Holder pasteurization was confirmed in the present study. These observations fit with the mechanism of human milk protein digestion proposed by Zhang et al. (2014), where proteins inside the casein micelle are less accessible for gastric digestion, whereas whey proteins and proteins at the surface of these structures are more accessible.

The absence of intact proteins in the intestinal compartment and the observed appearance of peptides suggest that the proteins emptied from the gastric compartment were rapidly hydrolyzed by the pancreatic proteolytic enzymes. The peptides were observed on the lower part of the gel, and the difference between peptides from RHM or PHM digestion is currently being investigated. Regarding the AA release, pasteurization modulated the bioaccessibility of some AA over intestinal digestion without modifying the total α-amino nitrogen released. For both RHM and PHM, a very low percentage of proline was released (2 – 3%) during intestinal digestion, which was likely due to the absence in our model of brush border peptidases such as aminopeptidase N and dipeptidyl peptidase IV (Le Huërou-Luron, 2002). To our knowledge, there is no data about the digestive AA release from human milk in term newborns. Thus, our set of data is crucial to get a better understanding of the human milk protein digestive kinetics, which is directly linked to their metabolic utilization and consequently plays an essential role in early nutrition (Lacroix et al., 2006).

It has been demonstrated that some lipolysis occurs in fresh human milk during refrigeration storage (Bertino et al., 2013; Slutzah, Codipilly, Potak, Clark, & Schanler, 2010; Lavine & Clark, 1987), and more extensively after storage at −20 °C associated with a freeze–thaw cycle (Bitman et al., 1983). Indeed, Hamosh (1983) reported that BSSL dependency on bile salts was reduced after storage at −20 °C. The action of endogenous lipases of the human milk is supposed to have positive effect on newborn nutrition since it improves lipid digestion. This natural potential for fat pre-digestion is particularly important in a context of digestive immaturity, in preterms and in the first weeks of life for instance (Pamblanco et al., 1987). Hence the pre-lipolysis of the undigested human milk used in our study is certainly due to the action of endogenous lipases of human milk (*i.e.* BSSL mainly and lipoprotein lipase) after the storage (until digestion for RHM and until pasteurization for PHM) associated to the freeze–thaw cycles. It should be noted that in our study RHM and PHM were both submitted to two cycles of freeze–thaw (after milk expression and after pasteurization). Considering PHM, we applied exactly the same steps of storage and heat treatment used in human milk banks. Thus, if BSSL is inactivated by heat treatment in pasteurized human milk, it can be active before pasteurization in the presence of low amount of bile salts typically reported in human milk (Bourlieu et al., 2014), and contributes to enhance milk fat digestibility.

Pasteurization limited lipolysis in HM before and during digestion. The gastric instantaneous lipolysis levels (18 – 24% of total acyl chains) found during RHM gastric digestion are in line with those reported by Roman et al. (2007) for preterm newborns (18 – 23% of total acyl chains). It is the first time that the rates of lipolysis are reported for PHM digestion. The inactivation of BSSL by pasteurization of human milk has been reported by various authors (Lindquist & Hernell, 2010; Henderson et al., 1998; Hamosh, 1983) and is a key element to explain the observed differences in digestive lipolysis between RHM and PHM. Another complementary effect contributing to this limitation could be the protein aggregation around the milk fat globule membrane induced by pasteurization, which could limit the lipase access to the triglyceride core and delay the lipolysis, as previously suggested (Armand et al., 1996). However, if this was the case, the initial difference in pre-lipolysis between the two milks would increase at least until the beginning of the intestinal phase, as pancreatic lipase is highly affected by interfacial quality (Bakala N'Goma et al., 2012); this was not observed. The limited impact of the quality of the interface in the gastric phase is in line with recent results from Bourlieu et al. (2015b), who demonstrated that the specific surface area of the milk emulsion is a predominant factor, affecting its kinetics of hydrolysis and its disintegration during gastric digestion. In our case, RHM and PHM had similar specific surfaces at the beginning of the digestion. Thus, it suggests that the initial difference in the activity of the BSSL before digestion was the main factor responsible for the more efficient lipid digestion of RHM.

Regarding the lipolysis products, the global evolution of lipid classes was little affected by the pasteurization, which had no impact on the profile of released FA; these results confirmed that the human milk lipid hydrolysis was highly dependent on the lipase specificity. The pre-lipolysis catalyzed by BSSL, which can hydrolyze any of the three positions of the TG, leads to FFA and glycerol release but can transiently generate DG and MG (Lindquist & Hernell, 2010). Afterwards, the gastric lipase, which is a *sn*-3 regioselective enzyme, generates mainly *sn*-1,2 DG and FFA (Bakala N'Goma et al., 2012). The presence of MG in the intestinal phase is in line with the regiospecificity of pancreatic lipase, which hydrolyzes *sn*-1,3 positions of TG molecules, generating *sn*-1,2(2,3) DG, *sn*-2 MG and FFA (Armand, 2008; Carrière et al., 1997). Also contributing to the intestinal lipolysis, the action of the human milk BSSL (still present in RHM) could be detected on the lipid classes as more MG were detected in PHM compared to RHM from 60 to 120 min of intestinal digestion. It can be explained by the very broad substrate of BSSL specificity and ability to hydrolyze MG in micellar or soluble form liberating glycerol and FFA as final products. The limited activity of BSSL observed in the intestinal phase *in vitro* is probably not true *in vivo*, where *sn*-2 MG and FFA can be progressively absorbed despite limiting bile salt concentrations (Bernback et al., 1990). Several *in vivo* studies have underlined the key role of BSSL in favoring fat absorption (Andersson et al., 2011) and clinical trials are being undertaken in Europe

and in the United States to examine the effects of additional BSSL in pasteurized human milk (Lonnerdal, 2013).

The increased mode diameter of the milk fat globules in PHM compared to RHM is probably linked to the reorganization of proteins around the milk fat globules, as observed by CLSM. The persistence of native milk fat globules over the gastric digestion is consistent with a partial lipolysis dominated by acid aggregation of the emulsion, and was already demonstrated *in vitro* in static or semi-dynamic models (Bourlieu et al., 2015b; Gallier, Ye, & Singh, 2012), and *in vivo* in preterm infants (Armand et al., 1996). Similarly, the greater mix of apolar (TG) and polar compounds (FFA, phospholipids) observed in the core of milk fat globules most for RHM gastric digestion, is in line with the higher extent of lipolysis compared to PHM. The structural instability observed after 60 min of RHM gastric digestion (and not PHM) did not affect the gap of lipolysis between the two types of meal, probably because lipolysis was already leveling off due to product inhibition (Pafumi et al., 2002). However, the difference in the physical behavior of these two emulsions may affect *in vivo* the rate of emptying and result in non-homogeneous emptying from the stomach to the intestine. It has been demonstrated (Golding et al., 2011; Marciani et al., 2007) that emulsions that are destabilized in the gastric compartment can lead to the faster emptying of the aqueous phase and consequently to a fat and energy accumulation, modulating the hormonal feedback (cholecystokinin). This may play an important role in the absorption of nutrients in the upper part of the intestine and global nutrient metabolism in infants. These two aspects could not be considered in our dynamic model: the same rate of gastric emptying was used for RHM and PHM (since there are no *in vivo* data about the rate emptying of pasteurized human milk); moreover, the absorption and hormonal feedback were not simulated.

Newborns present specific differences in the digestion process as compared to older infants or to adults, and term newborns present different digestive maturity compared to preterm (Bourlieu et al., 2014; Shani-Levi et al., 2013; Dupont et al., 2010a; Lindquist & Hernell, 2010). The input parameters proposed here have integrated these specificities since they were based on *in vivo* data and since some have been additionally validated in *in vitro* systems (Ménard et al., 2014). Thus, despite some limitations inherent to most *in vitro* systems, our model appears to be relevant to investigate human milk digestion in the term newborn.

The nutritional impact of different kinetics of liberation of hydrolysis products over the gastrointestinal digestion is ambiguous and has not been studied adequately. In one hand, quick digestion of some proteins may assure earlier bioavailability of nutriments or reduce allergenicity (Cordle, Mahmoud, & Moore, 1991; Dallas, Underwood, Zivkovic, & German, 2012). In the other hand, several studies have discussed the beneficial role of conveying intact or partially digested bioactive proteins through the

gastrointestinal tract (Chatterton et al., 2004; Dallas et al., 2012; Labbok et al., 2004). Notably, lactoferrin and some of its released peptides (*i.e.* lactoferricin) play multiple beneficial biological properties, *i.e.* antimicrobial, anti-inflammatory, immunomodulatory, regulatory of neonatal iron absorption (Garcia-Montoya et al., 2012; Lonnerdal, 2009; Hamosh, 2001; Bellamy, Takase, Wakabayashi, Kawase, & Tomita, 1992). The fact that pasteurization increases lactoferrin gastric hydrolysis could modify its biological activities, in particular by modulating the pattern of the bioactive peptides released. This should be further investigated. The lipolysis products released during human milk digestion are also well-known for their potential positive metabolic impacts. For instance, components such as lysosphingomyelin, sphingosine and medium chain FFA may have antimicrobial and probiotic activities (Bourlieu & Michalski, 2015). Moreover, long-chain polyunsaturated FA (particularly presents in human milk) play an essential role in infant growth and neural development (Innis, Gilley, & Werker, 2001).

Several studies have recently underlined the importance of collection and storage conditions on the preservation of the quality of human milk (Borgo et al., 2014; Penn et al., 2014; Vazquez-Roman, Alonso-Diaz, Garcia-Lara, Escuder-Vieco, & Pallas-Alonso, 2014). Some authors have proposed alternatives to minimize the side effects of these preservation processes (heat treatment, freeze–thaw cycles) without putting at risk the microbiological safety of the human milk from banks. For instance, Thomaz et al. (1999) proposed that homogenization applied after the pasteurization could reduce the side effects of pasteurization by restoring the lipolysis. More recently, ultraviolet-irradiation (Christen et al., 2013a; Christen et al., 2013b) and thermosonication (Czank, Simmer, & Hartmann, 2010) of human milk were proposed as substitute for Holder pasteurization. However, more studies are necessary to determine the impact of each of these technological treatments on the human milk digestion and assimilation.

Though some impacts of pasteurization on the human milk digestive behavior were evidenced in our study, we can globally consider that most beneficial components remain unchanged or little modified by the pasteurization and have protective function for infants (Wight, 2001). In our study for instance, there were similar FA profiles released from PHM or RHM. Infant formulas composition has been largely optimized but cannot provide all the range of bioactive components present in human milk. Indeed, the physical structure of fat droplets and their interfacial composition still differ a lot between human milk and infant formulas (Michalski, Briard, Michel, Tasson, & Poulain, 2005). These two parameters strongly modulate the efficiency of digestion and nutrients assimilation (Ye, Cui, & Singh, 2010; Roman et al., 2007; Armand et al., 1996). Therefore, even after pasteurization, the unique composition and demonstrated benefits of human milk makes this complex and evolving biological fluid a good choice for early nutrition (Boyd et al., 2007; Schanler et al., 2005). Moreover, this

pasteurized human milk can be fortified to make its nutritional content more adequate to the need of preterm infant.

2.1.6. Conclusion

The here proposed dynamic *in vitro* system gives access to parameters hardly accessible *in vivo, i.e.* the hydrolysis kinetics of lipids and proteins in the gastric and intestinal compartments as well as the concomitant structural changes. The results of this study expand the knowledge and understanding of human milk digestive behaviors, which is important for newborn health and development. Raw human milk is the ideal food for newborns, but pasteurized human milk from milk banks is a possible and well-accepted substitute. In this study, we showed that Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under simulated *in vitro* dynamic term newborn digestion. However, this impact is limited and its physiologic and metabolic consequences remain unknown. We underlined the importance of rethinking all the storage processes of human milk in order to optimize the preservation of the milk composition and structure. In the light of the actual knowledge, Holder pasteurized human milk remains certainly more beneficial than infant formulas for newborns, in particular for preterms. The impact of human milk pasteurization should also be investigated with an *in vitro* model of preterm digestion as they receive more often pasteurized human milk from banks than term newborns.

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2.2. Impact of pasteurization of human milk on preterm newborn *in vitro* digestion: Gastrointestinal disintegration, lipolysis and proteolysis

2.2.1. Abstract

Human milk feeding is an important recommendation for preterm newborns considering their vulnerability and digestive immaturity. Holder pasteurization (62.5 °C, 30 min) applied in milk banks modifies its biological quality and its microstructure. We investigated the impact of pasteurization of preterm human milk on its gastrointestinal kinetics of lipolysis, proteolysis and structural disintegration. An *in vitro* dynamic system was set up to simulate the gastrointestinal digestion of preterm newborns. A pool of preterm human milk was digested as raw or after Holder pasteurization. Pasteurization impacted the microstructure of undigested human milk, its gastrointestinal disintegration and tended to limit the intestinal lipolysis. Furthermore, the gastrointestinal bioaccessibility of some fatty acids was decreased by pasteurization, while the intestinal bioaccessibility of some amino acids was selectively modulated. The impact of pasteurization on the digestion of human milk may have nutritional relevance *in vivo* and potentially modulates preterm development and growth.

Keywords: Holder pasteurization; human milk; *in vitro* digestion; lipolysis; proteolysis; preterm newborn.

2.2.2. Introduction

Digestion of nutrients in newborns is essential for their optimized growth and development, but some of their digestive functions are immature at birth (Lindquist & Hernell, 2010; Hamosh, 1983). Preterm newborns are even more vulnerable and immature than term newborns of the same postnatal age, presenting higher gastric pH, lower enzymatic activities, faster gastric emptying, lower concentrations of electrolytes in digestive fluids, among other limitations (Bourlieu et al., 2014). According to the WHO (2012), 15 million babies were born prematurely in 2010 (*i.e.* 11% of livebirths before 37 weeks of gestation), and this rate is increasing every year. Preterm birth is a direct risk and leading cause of newborn mortality, besides increased risk for long-term physical, neuro-developmental and behavioral outcomes (Blencowe et al., 2013).

Considering their vulnerability and digestive constraints, human milk is a dynamic fluid that contains various bioactive components and facilitates the adaptation of newborns to extra-uterine life (Bourlieu et al., 2015a; Lonnerdal, 2014; Hamosh, 1983). For instance, some human milk enzymes modulate the digestion, such as bile-salt stimulated lipase (BSSL) favoring triglycerides hydrolysis and vitamins

absorption. Moreover, protease inhibitors such as α_1-antitrypsin and α_1-antichymotrypsin may limit the digestion of some beneficial bioactive proteins that compensate for developmental immaturity of the intestine (Dallas et al., 2012; Goldman, 2000). Therefore, human milk feeding is an important recommendation on intervention strategies to improve preterm birth outcomes (WHO, 2015).

Because of improved outcomes compared to infant formulas, *i.e.* on tolerance feeding, preventing inflammation and infections (Quigley & McGuire, 2014; Schanler et al., 2005), pasteurized human milk from milk banks is the second choice after fresh milk from the own mother (Picaud, 2015; Arslanoglu et al., 2013). However, as extensively reported, processing (collection, freeze-thaw cycles) and pasteurization of human milk modify its nutritional and biological quality (O'Connor et al., 2015) and its microstructure (de Oliveira et al., 2015). Indeed, bioactive nutrients such as BSSL, lactoferrin, oligosaccharides, immunoglobulins, iron, folate, vitamin C are reduced or inactivated after Holder pasteurization (O'Connor et al., 2015). These alterations may affect the paradoxical equilibrium of the human milk components (enzymes favoring digestion *versus* anti-proteases favoring the resistance of some components to digestion) (Goldman, 2000) and likely modulate human milk digestion.

It has been recently demonstrated that Holder pasteurization of term human milk has structural and biochemical consequences on its *in vitro* dynamic digestion at the term newborn stage (Deglaire et al., 2016; de Oliveira et al., 2015). Whether this remains true in preterm conditions (preterm human milk and preterm digestive parameters) is unknown. Due to ethical constraints limiting *in vivo* trials, relevant *in vitro* models of digestion are useful tools to investigate this question. The objective of this study was thus to determine the impact of Holder pasteurization of preterm human milk on its gastrointestinal digestion, using an original *in vitro* dynamic preterm newborn digestion model.

2.2.3. Materials and methods

Materials

Unless stated otherwise, chemicals are from commercial origin (Sigma-Aldrich, Saint Quentin Fallavier, France).

Human milk samples

Preterm mature human milk samples were obtained frozen from the donor milk bank of the University Hospital Center in Rennes (France), after ethical approval granted by the Hospital Ethics Committee (no. 13-12). Informed written consent was given by the five donors, aged of 26 to 34 years. Milk samples were expressed on average 6.6 ± 2.4 weeks after preterm delivery (range: 4 to 10 weeks). The conditions of collection, storage, pool and pasteurization of preterm human milk were previously detailed by de Oliveira et al. (2015). Briefly, after thawing and pooling, half of the pool remained raw

(raw human milk, RHM) and the other half underwent Holder pasteurization (62.5 °C, 30 min) (pasteurized human milk, PHM). Both RHM and PHM were stored at -20 °C until digestion. The macronutrients composition of the pool was assessed using a Human Milk Analyzer (Miris AB, Uppsala, Sweden), calibrated using the reference methods (Billard et al., 2015).

In vitro dynamic digestion

RHM and PHM were submitted to gastrointestinal digestion using the *in vitro* dynamic system DIDGI® (Ménard et al., 2014). This system was carefully set up to simulate human milk digestion in preterm four weeks old newborns (**Table 7**), based on an exhaustive review of *in vivo* digestive conditions (Bourlieu et al., 2014). The rationale for this selection was previously detailed (de Oliveira et al., 2015). The specific preterm stage parameters are described below. Gastric emptying rate followed the mathematical model described by Elashoff, Reedy & Meyer (1982) and was determined by fitting data from preterm newborns fed human milk (Ewer, Durbin, Morgan, & Booth, 1994). The estimated half-time ($t_{1/2}$) of gastric emptying was 36 min. Amounts of added gastric enzymes (from rabbit gastric extract) and bile salts (from bovine bile) were determined as a function of the mean body weight, estimated at 1.9 kg for preterm newborns with a gestational age of 28 weeks and a postnatal age of four weeks (Fenton, 2003). The compositions of simulated gastric and intestinal fluids and the amount of pancreatic lipases (from porcin pancreatin) were derived from data obtained in preterm newborns (Bourlieu et al., 2014; Fredrikzon & Olivecrona, 1978). Considering the amount of pancreatin added to cover the need of pancreatic lipase, the resulting amounts of trypsin and chymotrypsin used in the model were 105 and 15 U/mL, respectively.

Digestion experiments were performed in triplicate for each matrix, over 3 hours. Aliquots were collected before digestion and at 30, 60, and 90 min after the beginning of the milk ingestion from both gastric and intestinal compartments. Additional samples were collected from the intestinal compartment at 120 and 180 min. As described previously (de Oliveira et al., 2015), structural analyses were immediately performed. Samples for lipid analysis were either immediately submitted to lipid extraction before storage at -20 °C for thin layer chromatography and free fatty acids (FA) analyses or frozen at -20 °C for total FA analysis. Samples used for subsequent protein analysis were mixed with phenylmethylsulfonyl fluoride (PMSF) and then frozen at -20 °C.

Structural characterization

The microstructure of human milk and digesta was observed using confocal laser scanning microscopy (CLSM) on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) and a laser light scattering with two laser sources Mastersizer 2000 (Malvern Instruments, Malvern, UK), as previously

described in (Bourlieu et al., 2015a). The mode diameter, the volume-weighted average diameter of fat globules (D [4,3]) and the specific surface area were calculated.

Table 7. Gastrointestinal parameters for *in vitro* dynamic digestion of human milk simulating preterm conditions

Gastric conditions (37 °C)		
SGF	Na^+	118 mmol/L
(stock solution adjusted at pH 6.5)	K+	9.8 mmol/L
	Cl^-	137 mmol/L
Fasted state / initial conditions	SGF	2 mL
	pH	2.7
Milk ingested	Total volume	100 mL
	Flow rate	10 mL/min from 0 to 10 min
Gastric pH (acidification curve)	pH = - 0.0155*t + milk pH	
	with *t*: time after ingestion in min	
SGF + enzymes (RGE)	Gastric lipase	8.6 U/mL of gastric content
	Pepsin	120 U/mL of gastric content
	Flow rate	1 mL/min from 0 to 10 min
		0.5 mL/min from 10 to 180 min
Gastric emptying	$t_{1/2}$	36 min
(Elashoff fitting)	β	1.15
Intestinal conditions (37 °C)		
SIF	Na^+	140 mmol/L
(stock solution adjusted at pH 6.2)	K^+	4 mmol/L
	Ca^{2+}	41 mmol/L
Intestinal pH	6.2	
SIF + bile	Bile salts	1.6 mmol/L of intestinal content
	Flow rate	0.5 mL/min from 0 to 180 min
SIF + pancreatin	Pancreatic lipase	59 U/mL of intestinal content
	Flow rate	0.25 mL/min from 0 to 180 min
Intestinal emptying	$t_{1/2}$	200 min
(Elashoff fitting)	β	2.2

RGE, rabbit gastric extract; SGF, simulated gastric fluid; SIF, simulated intestinal fluid

Lipid analysis

Three complementary lipid analyses were conducted on milk and digesta: analysis of lipid classes (residual triglycerides, diglycerides, monoglycerides and free FA) by thin layer chromatography coupled to a flame ionization detector (IATROSCAN MK5 equipment, Iatron Laboratories, Tokyo, Japan); analysis of free FA (C8:0 to C18:3) by gas chromatography after SPE; and total esterified FA (C8:0 to C24:0) by gas chromatography coupled to a flame ionization detector. The first two analyzes were performed on the chloroform phase recovered after direct Folch extraction, and the last one after direct transmethylation. These analyses were previously described by de Oliveira et al. (2015).

The instantaneous lipolysis degree during digestion was expressed at a given time as the percentage level of free FA (in moles) versus the total acyl chains present in residual glycerides. The relative

contents of each FA methyl ester were expressed as mass % and used to calculate individual FA bioaccessibility: the FA released during digestion was expressed as the percentage level of free FA versus the corresponding total FA present in the undigested milk. The level of free FA was expressed in g/100 g of human milk by dividing the digesta FA content by the proportion of residual human milk in this digesta.

Protein analysis

SDS-PAGE analyses and image analyses of the gels, total and free amino acid (AA) analyses were performed as previously detailed by de Oliveira et al. (2015). After densitometry analyses of the gels, the percentage of each intact protein remaining in the gastric compartment at a given time was estimated in comparison with the undigested human milk. The AA bioaccessibility was expressed as the percentage level of free AA versus total AA, calculated as detailed above for FA bioaccessibility.

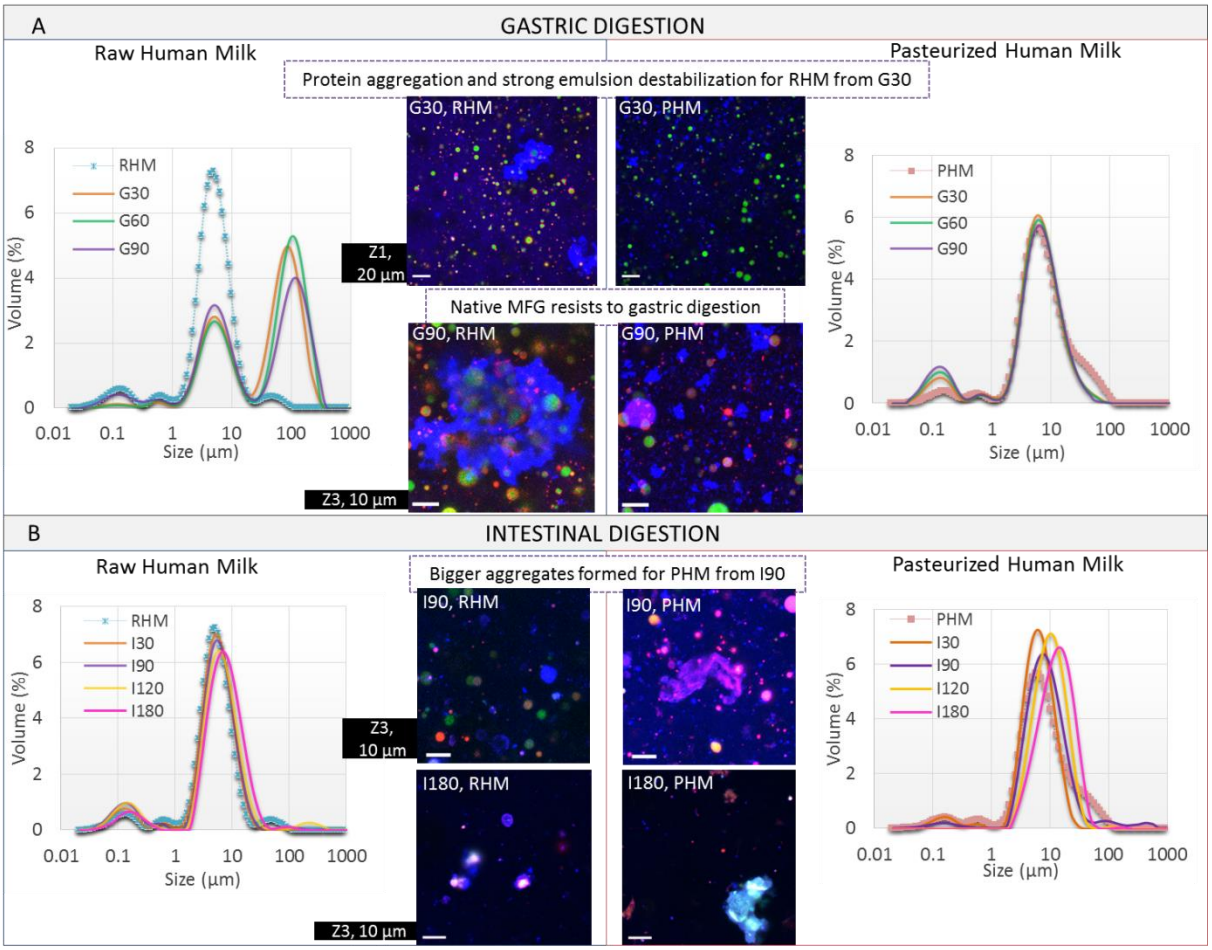
Statistical analysis

The differences between groups were tested by analysis of variance for repeated measures using the MIXED procedure with time as repeated factor under the SAS software (SAS 9.4; SAS Institute Inc., Cary, NC). For each parameter, eight different covariance structures for random statements [Unstructured, Compound Symmetry, Heterogeneous Compound Symmetry, Auto Regressive (1), Heterogeneous Auto Regressive (1), Auto Regressive Moving Average (1,1), Spatial Power and Toeplitz] were tested, and the most appropriate matrix was selected based on the fit statistics (AIC and BICC criterion). Post hoc tests were performed using contrast analysis. A p-value < 0.05 was considered as statistically significant. Results were expressed as means ± SD.

2.2.4. Results

Preterm mature human milk is modified by pasteurization

Holder pasteurization impacted the structure of the preterm human milk (**Figure 19** and **Supplementary Figure 1**), mainly through protein aggregation in the soluble phase and around the milk fat globule membrane. This aggregation resulted in an increase of the mode diameter (5.2 ± 0.0 and 6.3 ± 0.1 µm in RHM and PHM, respectively) and of the D [4,3] (7.3 ± 0.5 and 12.8 ± 1.7 µm in RHM and PHM, respectively), and in a decrease of the specific surface (5.0 ± 1.3 and 3.0 ± 1.0 m^2/g fat in RHM and PHM, respectively). PHM presented a higher contribution of large aggregates between 20 and 100 µm. After laser light scattering in dissociating agent (SDS), these large aggregates were still present in PHM but not in RHM (data not shown).



C EVOLUTION OF SIZE PARAMETERS

		HM	G30	G60	G90	Meal	Time	Meal* time	I30	I90	I120	I180	Meal	Time	Meal* time
Mode (µm)	RHM	5.2 ± 0.0	96.3 ± 24.5^{b}	114.8 ± 4.5^{b}	124.7 ± 15.9^{b}	**	***	***	5.5 ± 0.2	5.7 ± 0.1	6.1 ± 0.2^{b}	7.2 ± 0.2^{b}	**	**	*
	PHM	6.3 ± 0.1	6.2 ± 0.1^{a}	6.3 ± 0.1^{a}	6.5 ± 0.3^{a}				6.3 ± 0.4	8.0 ± 1.3	10.7 ± 1.9^{a}	14.5 ± 1.9^{a}			
D [4, 3] (µm)	RHM	7.3 ± 0.5	65.2 ± 17.1^{b}	79.1 ± 4.8^{b}	71.7 ± 14.0^{b}	***	***	***	6.3 ± 0.5	7.9 ± 2.2^{b}	12.6 ± 7.6	9.6 ± 0.1^{b}	NS	***	**
	PHM	12.8 ± 1.7	8.6 ± 0.1^{a}	8.5 ± 0.1^{a}	8.2 ± 0.3^{a}				7.3 ± 0.9	17.7 ± 2.2^{a}	12.6 ± 2.7	16.1 ± 0.8^{a}			
SS (m^2/g fat)	RHM	5.0 ± 1.4	1.5 ± 0.2^{b}	1.3 ± 1.1^{b}	4.1 ± 1.0^{b}	**	***	***	6.0 ± 1.6	7.3 ± 1.2^{b}	6.5 ± 0.7^{b}	4.4 ± 2.6	0.07	*	*
	PHM	3.0 ± 1.0	6.1 ± 0.1^{a}	7.3 ± 1.1^{a}	8.4 ± 0.5^{a}				3.4 ± 1.0	1.9 ± 1.6^{a}	0.7 ± 0.1^{a}	0.6 ± 0.0			

Figure 19. Disintegration of preterm RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic preterm newborn digestion, and evolution of the associated size parameters (C). Images are from CLSM (two observations, x60 zoom 1 and 3). Proteins are colored in blue (FastGreen®), apolar lipids in green (Lipidtox®) and polar lipids in red (Rhodamine-PE). Particle size distribution and size parameters were determined by laser light scattering in water. Data represents the average of two digestions, each of them being measured in triplicate. G, gastric phase followed by time in min; HM, human milk; I, intestinal phase followed by time in min; MFG, milk fat globule; SS, specific surface. Statistics were conducted on size parameters from HM to G90 and from I30 to I180. $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.1$ (NS). When there was a significant interaction, multiple comparisons of means were conducted between RHM and PHM. Data having a different letter within the same parameter and at the same time point differed significantly.

Pasteurization did not impact macronutrient composition, total FA and AA profiles (**Supplementary Figure 1**). Preterm RHM and PHM presented 35.7 ± 0.7 g/L of fat, 13.2 ± 0.8 g/L of proteins and 78.3 ± 2.3 g/L of carbohydrates. Concerning the total FA, 50.8% were saturated and 49.2% unsaturated. The ratio ω-6/ω-3 was 10.1. Total and free AA contents of undigested preterm RHM and PHM are

displayed in **Supplementary Table 1**. Total AA was dominated by glutamic acid, followed by leucine and aspartic acid. Glutamic acid was again the most present AA in the free form (15.8% of total AA), followed by alanine and glycine (4.8 and 2.2% of total AA, respectively).

Pasteurization impacts the gastrointestinal disintegration of preterm mature human milk

Figure 19 shows the CLSM images and the evolution of particle size distribution of preterm RHM and PHM during gastric (A) and intestinal (B) digestion together with the associated particle size parameters D [4,3], specific surface and mode (C). A strong emulsion destabilization was observed for RHM from 30 min of gastric digestion onwards, with the formation of large aggregates resulting in a bimodal distribution of the particle size (**Figure 19**A), an increase in the main mode diameter and in the D [4,3], and a decrease in the specific surface (**Figure 19**C). The large aggregates observed in the CLSM images were likely due to particle aggregation, since the mode diameter of undigested RHM was quasi-restored after measurements in SDS (data not shown). For PHM, the aggregates observed between 20 and 100 µm in undigested PHM disappeared from the beginning of the gastric digestion (**Figure 19**A) with a subsequent decrease in the D [4,3] and an increase in the specific surface (**Figure 19**C). Afterwards, the size parameters did not evolve through the entire gastric digestion, except for specific surface which increased slightly from 6.1 to 8.4 m^2/g (**Figure 19**C).

In the intestinal phase, larger aggregates were formed for PHM than for RHM. The mode diameter and the D [4,3] increased over time for both matrices, slowly for RHM and more intensely for PHM (**Figure 19**C). This increase was inversely proportional to the decrease in specific surface.

Lipolysis kinetics and fatty acid release

Figure 20 shows the kinetics of lipolysis of preterm RHM and PHM. There was no significant difference ($p > 0.05$) of lipolysis degree between RHM and PHM before and during gastric digestion (**Figure 20**A). The degree of lipolysis increased over time for RHM and PHM, from 3.0 ± 0.5% before digestion up to 13.1 ± 1.4% at 90 min of digestion. During the intestinal digestion (**Figure 20**B), the lipolysis degree tended to be significantly higher for RHM than for PHM ($p = 0.056$). The intestinal lipolysis degree sharply increased for both RHM and PHM between 30 and 180 min, with values of 21.0 ± 2.1% up to 53.6 ± 1.9% for RHM and of 16.6 ± 2.0% up to 51.0 ± 1.5% for PHM, respectively.

The evolution of the lipid classes over digestion (acyl chains mol%) was linked to the evolution of the lipolysis degree: quite stable over the gastric phase and emphasized by progressive decrease of triglycerides and the increase of lipolysis products in the intestinal phase. Monoglycerides were detected only in the intestinal phase and their content was higher in PHM than in RHM from 90 to 180 min (**Supplementary Figure 2**).

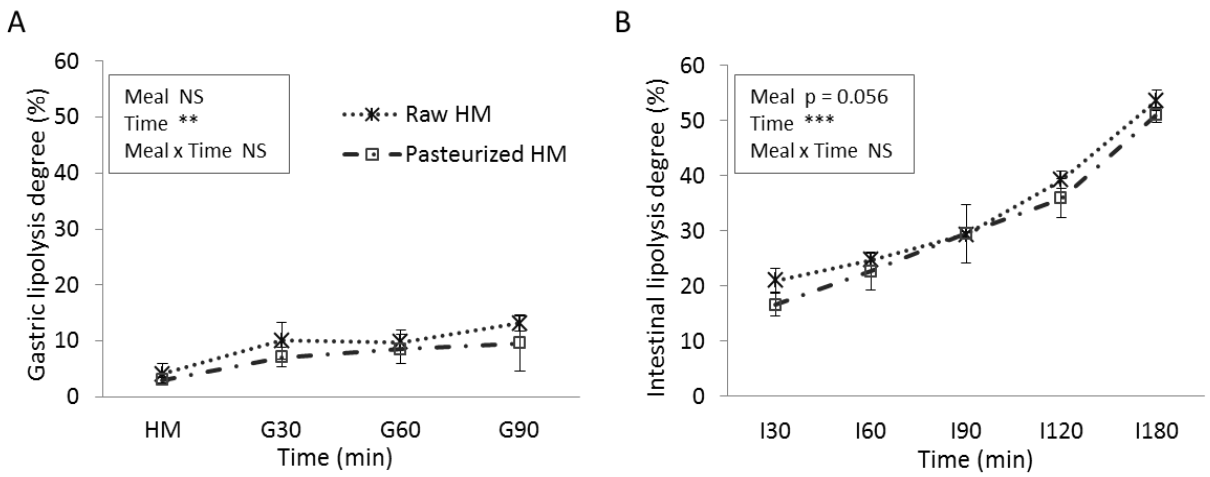


Figure 20. Kinetics of lipolysis of RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic digestion simulating newborn preterm conditions. The lipolysis degree (% of FA released from total initially esterified FA) was determined based on thin layer chromatography coupled to a flame ionization detector for digesta and on gas chromatography for undigested milk. Data point represents means ± SD (n = 3 for each milk). $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.1$ (NS). G and I, respectively gastric and intestinal phases followed by time in min.

When comparing the released FA with the acyl chains initially esterified in human milk (**Supplementary Figure 3**), it appeared that oleic acid (C18:1 c9) and stearic acid (C18:0) were selectively released, as their relative amount were higher than that in the total esterified FA profile. The level of released FA from RHM and PHM increased significantly during gastric and intestinal digestion (**Table 8**) and was individually impacted by pasteurization. The percentages of released C8:0, C10:0, C12:0 and C14:0 were significantly lower for PHM compared to RHM during the entire course of the gastric digestion and only at 90 min for C14:1 c9. The percentage of C18:2 c9,c12 tended to be lower in PHM compared to RHM at 90 min of the gastric digestion ($p = 0.08$). The amount of C18:3 c9,c12,c15 released during the gastric phase was not determined due to detection threshold. During the intestinal digestion, the percentages of most of the released FA were significantly lower in PHM compared to RHM, except for C14:1 c9, C16:1 c9 (trend, $p = 0.06$) and C18:0 ($p > 0.1$). This difference was observed during the entire course of the intestinal digestion (except for C14:1 c9, only at 180 min). Globally, more FA were released during the intestinal stage than during the gastric one. In both gastric and intestinal digestion, C8:0 presented the higher percentage of release.

Table 8. Fatty acid released from RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic preterm digestion

Fatty acid	A. FA released during gastric digestion (% *w/w* of each FA initially esterified)								Effect of [a)]		
	Human milk		G30		G60		G90		Meal	Time	Meal* Time
	RHM	PHM	RHM	PHM	RHM	PHM	RHM	PHM			
C8:0	56.2 ± 23.5	26.1 ± 1.6	67.2 ± 13.3	33.2 ± 3.1	78.4 ± 11.9	39.9 ± 8.1	94.9 ± 13.6	60.1 ± 1.0	**	**	NS
C10:0	15.5 ± 9.1	6.7 ± 1.1	26.1 ± 2.9	13.4 ± 3.1	33.4 ± 2.0	20.7 ± 4.5	44.8 ± 7.7	31.8 ± 6.7	**	*	NS
C12:0	7.1 ± 4.4	3.0 ± 0.3	9.6 ± 1.7	4.6 ± 0.6	12.2 ± 0.1	7.2 ± 1.9	16.3 ± 2.3	11.9 ± 2.1	**	*	NS
C14:0	2.9 ± 1.6	2.2 ± 0.2	3.7 ± 0.7	2.1 ± 0.2	4.5 ± 0	2.9 ± 0.6	6.3 ± 0.4	4.3 ± 0.1	*	**	NS
C14:1 c9	9.5 ± 5.1	4.8 ± 2.1	8.8 ± 4.3	2.8 ± 3.4	10.1 ± 1.0	3.7 ± 4.1	13.3 ± 1.8[b]	0 ± 0[a]	*	***	***
C16:0	2.8 ± 1.2	2.8 ± 0.7	3.7 ± 1.3	2.8 ± 0.6	4.8 ± 1.4	3.9 ± 0.6	7.7 ± 2.1	5.3 ± 0.9	NS	***	***
C16:1 c9	7.7 ± 3.7	4.7 ± 1.5	8.6 ± 4.5	3.7 ± 0.9	9.1 ± 4.4	4.3 ± 0.6	11.8 ± 5.2	5.4 ± 1.2	NS	*	NS
C18:0	7.8 ± 2.5	8.3 ± 0.4	9.0 ± 3.1	8.8 ± 0.6	13 ± 3.9	12.4 ± 1.4	21.0 ± 7.5	19.1 ± 1.8	NS	***	NS
C18:1 c9	5.1 ± 2.2	3.5 ± 0.9	5.3 ± 2.2	2.5 ± 0.6	5.6 ± 2.2	2.7 ± 0.1	6.8 ± 2.1[b]	3.2 ± 0.9[a]	NS	**	*
C18:2 c9,c12	5.5 ± 2.3	3.3 ± 1.0	6.2 ± 2.8	2.6 ± 0.5	6.4 ± 2.6	2.7 ± 0.3	7.4 ± 2.4[b]	3.3 ± 0.8[a]	0.08	***	**
C18:3 c9,c12,c15	ND	ND	ND	ND	ND	ND	ND	ND			

Fatty acid	B. FA released during intestinal digestion (% *w/w* of each FA initially esterified)						Effect of [a)]		
	I60		I120		I180		Meal	Time	Meal * Time
	RHM	PHM	RHM	PHM	RHM	PHM			
C8:0	79.5 ± 11.5	47.9 ± 7.0	92.5 ± 9.6	63.9 ± 12.3	105.3 ± 14.1	83.3 ± 5.1	*	***	NS
C10:0	43.7 ± 3.4[b]	28.0 ± 5.0[a]	60.2 ± 3.0[b]	46.5 ± 5.8[a]	87.5 ± 8.2[b]	65.7 ± 7.1[a]	*	***	*
C12:0	26.6 ± 2.5[b]	15.9 ± 3.5[a]	42.3 ± 3.2[b]	30.7 ± 3.8[a]	67.4 ± 6.7[b]	45.8 ± 6.9[a]	*	***	*
C14:0	14.8 ± 1.5[b]	9.5 ± 2.7[a]	27.1 ± 2.4[b]	21.1 ± 3.5[a]	47.8 ± 4.2[b]	33.6 ± 6.4[a]	*	***	*
C14:1 c9	25.2 ± 2.5[b]	15.4 ± 4.0[a]	39.3 ± 3.3	33.1 ± 3.6	62.2 ± 4.5	47.9 ± 10.1	0.06	***	*
C16:0	10.1 ± 1.4	7.6 ± 0.5	17.6 ± 2.0	14.2 ± 1.2	32.9 ± 5.4	23.0 ± 1.7	*	***	NS
C16:1 c9	19.7 ± 4.2	13.1 ± 1.0	31.3 ± 4.6	24.4 ± 1.2	50.9 ± 8.8	37.5 ± 3.1	0.06	***	NS
C18:0	23.1 ± 4.0	21.8 ± 1.2	39.4 ± 3.5	39.2 ± 0.9	68.7 ± 8.3	63.0 ± 3.2	NS	***	NS
C18:1 c9	16.1 ± 1.3	11.0 ± 0.8	28.0 ± 1.2	22.9 ± 1.9	46.2 ± 4.8	38.0 ± 1.8	*	***	NS
C18:2 c9,c12	17.2 ± 1.2	11.0 ± 0.5	29.3 ± 1.2	22.7 ± 2.3	48.4 ± 3.9	37.4 ± 2.9	**	***	NS
C18:3 c9,c12,c15	22.4 ± 0.8	8.6 ± 7.5	38.1 ± 2.0	25.9 ± 3.4	60.6 ± 2.4	43.6 ± 4.7	**	***	NS

G, gastric phase followed by time in min; I, intestinal phase followed by time in min; ND, not determined.

[a)] Statistics were conducted on data from human milk to G90 and from I60 to I180. $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.1$ (NS). When there was a significant interaction, multiple comparisons of means were conducted. Data having a different letter within the same row and at the same time point significantly differed.

Proteolysis kinetics and amino acids release

Figure 21 shows the disappearance of the major proteins lactoferrin, serum albumin, β-casein and α-lactalbumin (theoretical molecular weight without signal peptides of 76.30, 66.47, 23.86 and 14.08 kDa, respectively - UniProtKB/Swiss-Prot) during gastric and intestinal digestion of preterm RHM and PHM. The kinetics of the meal proportion remaining in the total digesta volume (meal + secretions), assumed to be the same for RHM and PHM, indicated whether the protein disappearance was only due to emptying and dilution by secretions or whether proteolysis occurred. In the gastric phase, proteolysis occurred to lactoferrin and β-casein (Figure 21C and E, respectively), whereas the disappearance of serum albumin and mainly α-lactalbumin (Figure 21D and F, respectively) followed the meal disappearance. Pasteurization did not significantly impact the kinetics of disappearance of the major proteins ($p > 0.05$).

Concerning the intestinal phase (Figure 21B), drastic proteolysis was observed. The unique intact protein from milk observed in the electrophoresis gel was lactoferrin, for which the signal was more intense for PHM than for RHM. The other bands visible on gels corresponded to the proteins from porcine pancreatin (~53 and 24 kDa). The signal of peptides arising from protein digestion (~2 to 6 kDa) was clearly less visible for RHM than for PHM at 90 and 120 min of the intestinal digestion.

The level of AA present in the free form did not significantly increase during the gastric digestion for both RHM and PHM (data not shown), but sharply increased during intestinal digestion ($p < 0.001$), as displayed in **Table 9**. The percentages of released phenylalanine, tyrosine and arginine were significantly higher for PHM compared to RHM during the entire course of the intestinal digestion ($p < 0.01$). In contrast, the percentage of released proline was lower in PHM compared to RHM (entire intestinal digestion, $p < 0.01$) such as for serine (at 60, 90 and 180 min, $p < 0.05$). More α-amino nitrogen was released from PHM than from RHM during the intestinal digestion (+ 11% on average, $p < 0.05$).

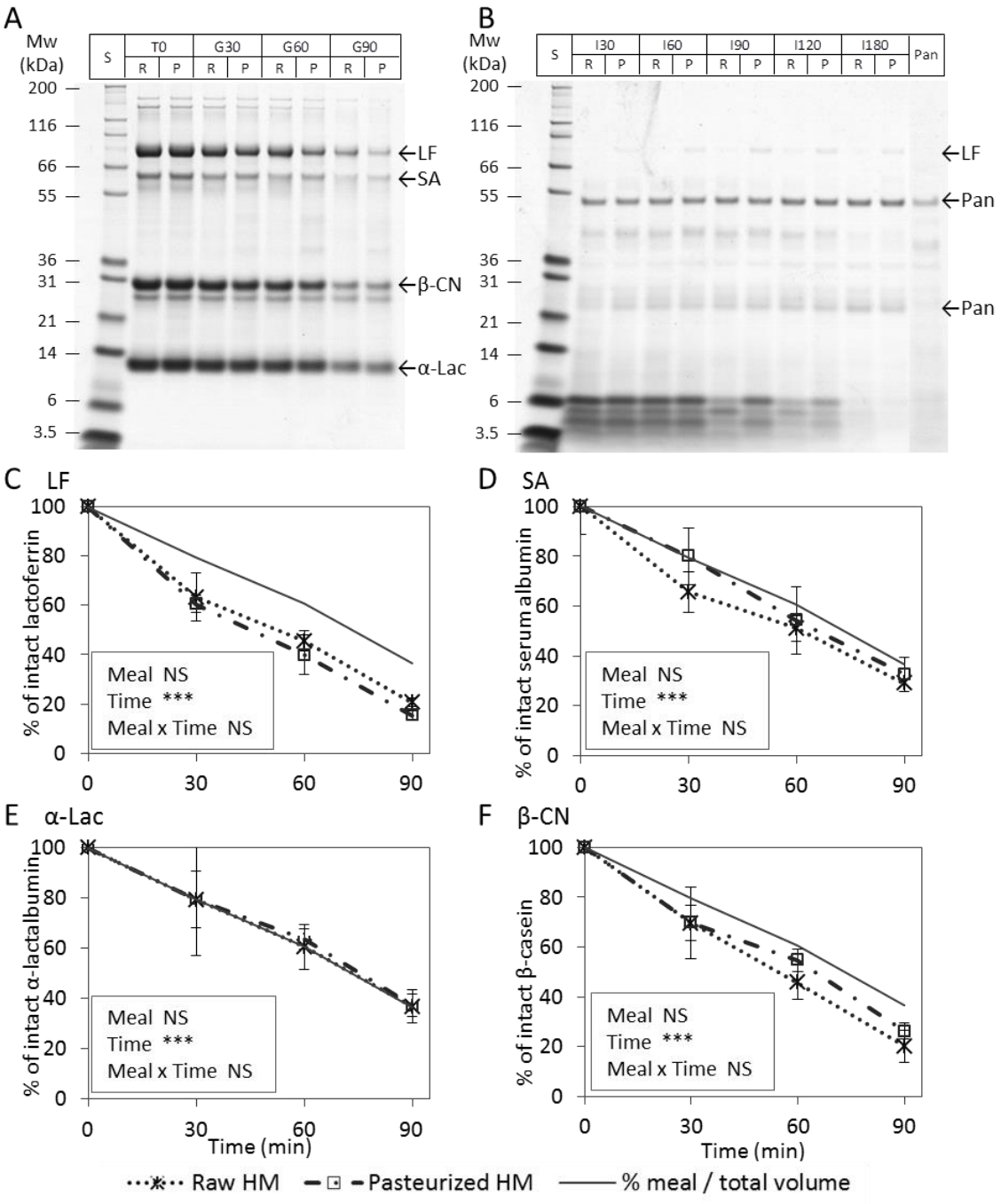


Figure 21. SDS-PAGE protein profiles of preterm RHM and PHM during gastric (A) and intestinal (B) *in vitro* dynamic preterm digestion. Raw (R) and pasteurized (P) preterm human milk digesta appear side by side on the gels for each time (left: R; right: P). Protein molecular mass standards (S) are on the left. Porcine pancreatin (Pan) diluted in SIF was used as control. Percentage of intact proteins during gastric digestion as obtained by densitometry for lactoferrin (LF) (C), serum albumin (SA) (D), a-lactalbumin (a-Lac) (E) and b-casein (b-CN) (F) and percentage of meal in the total volume at each time point. Data points in panels C to F represent means ± SD (n = 3 for each milk). $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.1$ (NS). Data from undigested milk were not included in the statistical analysis.

Table 9. Amino acids released from RHM and PHM during intestinal *in vitro* dynamic preterm digestion

Amino acid	Amino acid released during digestion (*% w/w* of total amino acids)												
	Human milk		I60		I90		I120		I180		Effect of [a)]		
	RHM	PHM	RHM	PHM	RHM	PHM	RHM	PHM	RHM	PHM	Meal	Time	Meal * Time
Isoleucine	0 ± 0	0 ± 0	4.9 ± 0.3	5.2 ± 0	7.5 ± 0.8	7.9 ± 0.3	12.1 ± 2.6	11.9 ± 0.5	24.4 ± 1.9	26.8 ± 2.4	NS	***	NS
Leucine	0.6 ± 0	0.6 ± 0	17.4 ± 0.1	18.5 ± 0.3	22.3 ± 0.9	24.1 ± 0.8	28.2 ± 2.7	30.2 ± 1.2	42.7 ± 1.4	46.7 ± 3.8	NS	***	NS
Lysine	0.2 ± 0	0.3 ± 0.1	22.7 ± 0.8	25.3 ± 0.2	29.4 ± 1.8	33.1 ± 0.6	38.4 ± 4.3	42.7 ± 0.6	65.0 ± 3.2	70.7 ± 3.0	NS	***	NS
Methionine	0 ± 0	0 ± 0	8.3 ± 0.2	7.3 ± 0.1	12.5 ± 0.7	12.0 ± 0.9	18.7 ± 2.3	17.7 ± 0.8	33.2 ± 1.7	34.4 ± 2.5	NS	***	NS
Cysteine	1.4 ± 0.2	1.6 ± 0.1	6.2 ± 1.2	4.8 ± 0.4	5.3 ± 0.2	5.1 ± 0.4	7.1 ± 0.5	6.4 ± 0.2	12.9 ± 1.9	13.7 ± 1.0	NS	***	NS
Phenylalanine	0 ± 0	0 ± 0	13.0 ± 0.7	19.1 ± 0.4	19.1 ± 1.1	27.0 ± 0.9	26.9 ± 3.0	35.6 ± 1.8	47.1 ± 1.7	56.7 ± 4.0	**	***	NS
Tyrosine	0.4 ± 0	0.4 ± 0	30.8 ± 0.3	37.6 ± 0.6	38.0 ± 1.9	46.8 ± 1.5	46.8 ± 4.1	57.2 ± 2.1	71.9 ± 2.8	83.8 ± 4.6	**	***	NS
Threonine	1.8 ± 0.2	1.8 ± 0.1	8.2 ± 0.1	7.1 ± 0.1	9.8 ± 0.6	9.0 ± 0.2	12.6 ± 1.6	13.8 ± 3.2	23.3 ± 1.2	24.2 ± 0.9	NS	***	NS
Valine	0.8 ± 0.1	0.8 ± 0	7.4 ± 0.5	6.8 ± 0.1	10.4 ± 0.7	9.8 ± 0.4	14.1 ± 1.8	13.5 ± 0.4	24.7 ± 1.1	24.2 ± 1.5	NS	***	NS
Histidine	1.7 ± 0.3	1.8 ± 0.1	8.1 ± 0.2	8.2 ± 0.1	8.2 ± 1.5	8.9 ± 0.9	12.0 ± 1.3	12.5 ± 0.9	23.9 ± 2.0	24.2 ± 1.7	NS	***	NS
Glutamic acid	15.9 ± 1.7	15.7 ± 1.2	20.1 ± 0.4	19.4 ± 0.4	21.9 ± 1.1	21.7 ± 0.4	25.0 ± 2.1	24.6 ± 0.5	37.3 ± 1.3	37.8 ± 1.9	NS	***	NS
Aspartic acid	1.0 ± 0.1	1.0 ± 0.1	6.0 ± 0.3	5.9 ± 0.2	7.4 ± 0.3	7.4 ± 0	9.7 ± 1.2	10.3 ± 0.3	18.9 ± 1.0[b]	20.2 ± 0.2[a]	NS	***	*
Proline	0.2 ± 0	0.2 ± 0	0.7 ± 0[a]	0.5 ± 0.1[b]	0.9 ± 0.1[a]	0.6 ± 0[b]	1.4 ± 0.2[a]	0.9 ± 0.1[b]	3.1 ± 0.4[a]	2.0 ± 0.2[b]	**	***	**
Serine	1.6 ± 0.1	1.7 ± 0.1	8.5 ± 0.1[a]	7.4 ± 0.1[b]	10.2 ± 0.6[a]	8.8 ± 0.1[b]	12.5 ± 1.2	11.6 ± 0.6	21.9 ± 1.5[a]	20.3 ± 2.0[b]	NS	***	*
Glycine	2.2 ± 0.1	2.3 ± 0.1	8.4 ± 0.2	8.1 ± 0	9.9 ± 0.6	10.0 ± 0.3	12.3 ± 1.1	12.3 ± 0.3	23.5 ± 1.1	23.7 ± 0.1	NS	***	NS
Alanine	4.8 ± 0.4	4.9 ± 0.2	11.6 ± 0.1	11.9 ± 0.2	14.2 ± 0.6	15.2 ± 0.5	18.1 ± 1.9	19.3 ± 0.2	31.9 ± 1.3	34.7 ± 1.5	NS	***	NS
Arginine	0 ± 0	0 ± 0	39.8 ± 0.8	50.6 ± 0.3	46.0 ± 2.5	59.2 ± 1.0	53.2 ± 4.8	69.2 ± 0.5	82.7 ± 4.5	99.1 ± 1.7	**	***	NS
α-amino nitrogen [b)]	3.3 ± 0.3	3.3 ± 0.2	12.6 ± 0.1	13.3 ± 0.2	15.4 ± 0.7	16.6 ± 0.4	19.4 ± 1.9	20.9 ± 0.6	32.1 ± 1.3	34.4 ± 1.6	*	***	NS

I, intestinal phase followed by time in min.

[a)] Statistics were conducted on data from I60 to I180. $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); $p > 0.05$ (NS). When there was a significant interaction, multiple comparisons of means were conducted. Data having a different letter within the same row and at the same time point significantly differed.

[b)] Calculated from the proportion of α-amino nitrogen in the free compared to the total amino acids.

2.2.5. Discussion

Using an original *in vitro* dynamic model to simulate preterm newborn digestion, we demonstrated that pasteurization impacted the gastrointestinal disintegration by inducing the formation of smaller gastric aggregates but larger intestinal ones in PHM, and also impacted the kinetics of digestion by decreasing the gastrointestinal release of some FA and modulating selectively the intestinal release of AA.

The pasteurization reduced the gastric destabilization: whereas an important destabilization was observed as soon as 30 min of gastric digestion for preterm RHM, PHM particle size profile remained quasi-steady during all the gastric phase. A similar result was found for the term digestive conditions with a destabilization of RHM from 60 min onwards (de Oliveira et al., 2015). Aggregates formed during RHM digestion presented slightly larger modes in the preterm (mode 1 = 96.3, mode 2 = 5.4 at 30 min) than in the term (mode 1=76.5 µm, mode 2= 6.5 µm at 60 min) conditions. As observed by CLSM presently and previously, these aggregates were based on clusters of milk fat globules around which protein aggregates built up (Bourlieu et al., 2015b; de Oliveira et al., 2015; Gallier et al., 2013b). Presenting lower density than milk aqueous phase, these lipoproteic clusters are prone to cream in the gastric compartment. Such gastric destabilization in the adult favors a rapid gastric emptying of the aqueous low caloric watery phase, whereas the creamy phase is emptied more slowly (Golding & Wooster, 2010). The early gastric destabilization of raw human milk observed here could be a protective mechanism favoring emptying and compensating the immature gastric motility in preterm newborns. Pasteurization, which limits such aggregation, could have negative physiological impact since delayed gastric emptying potentially increases the risk of gastrointestinal infections (Berni Canani & Terrin, 2010).

A pronounced negative impact of the pasteurization on the lipolysis degree was expected, as previously suggested (Armand et al., 1996; Hamosh, 1996; Lindquist & Hernell, 2010) and considering the measurement of the endogenous lipase activity (23.4 ± 3.3 U/mL in RHM and 0 U/mL in PHM as assessed by pH-stat and using tributyrin as substrate, data not shown). This pronounced impact was not observed on the instantaneous gastric lipolysis. However, our results revealed an impact on the quality of the released FA. The short to medium-chains FA (C8:0 to C14:1 c9) and the C18:2 c9,c12 acid were more released from RHM in the gastric phase, and are positioned mainly in the external positions (*sn-1,3*) of the glycerol backbone (Bourlieu & Michalski, 2015; Innis, 2011). Such positions correspond to the regioselectivity of the gastric lipase (*sn*-3) and of the BSSL (mainly *sn*-1 and *sn*-3), which suggest a concerted action of gastric lipase and BSSL in the RHM (Bernback et al., 1990). A larger impact was observed during the intestinal phase. Resistant to gastric digestion, BSSL from RHM is likely triggered by the presence of intestinal bile salts and then act in synergy with colipase-dependent pancreatic

lipase and gastric lipase, favoring the intestinal lipolysis of RHM comparing to PHM (Bakala N'Goma et al., 2012; Bernback et al., 1990).

The higher intestinal bioaccessibility of some FA in RHM observed in our study can have nutritional and clinical impacts for the preterm, since these FA have different luminal and systemic functions. For instance, medium-chain FA have a luminal bactericidal activity, playing a part in the establishment of the microbiota in the preterm otherwise very susceptible to gastrointestinal infections (Nejrup et al., 2015). Although less abundant compared to long-chain FA, short to medium-chain FA are known to be quickly assimilated *via* β-oxidation in the liver (Bourlieu & Michalski, 2015). Furthermore, long-chain PUFAs are recognized to be involved in brain development and are among the main components of the neuronal membrane. Indeed, preterm infants have high requirements of PUFAs and long-chain PUFAs for ensuring rapid tissue growth and optimal development (Collado et al., 2015). Therefore, the negative impact of pasteurization on the FA bioaccessibility potentially limits their beneficial effects.

Gastric proteolysis kinetics of the major proteins showed no difference between RHM and PHM. Here, the initial differences between undigested RHM and PHM were not taken into account but could have nutritional consequences. In the present preterm human milk, the signal of lactoferrin was three times lower in PHM than in RHM, as assessed by reverse HPLC (data not shown). This well-known denaturation of lactoferrin induced by Holder pasteurization (O'Connor et al., 2015) reduces its beneficial bioactive role in the gastrointestinal tract (increasing iron delivery and decreasing bacterial growth) (Lonnerdal, 2014). In the intestinal phase, we showed for the first time that pasteurization induced a greater release of α-amino nitrogen in preterms, although the extent of the difference remains small. The AA most released (arginine, tyrosine and phenylalanine) presented higher bioaccessibility in PHM compared to RHM. Understanding the pattern of AA intestinal bioaccessibility is an important step to better understand the protein metabolism of infants, which will determine their availability for protein synthesis. The impact of pasteurization on the AA bioavailability and the physiological consequences should be further investigated. Indeed, incomplete proteolysis seems to have beneficial functions. Various authors have suggested the persistence of some intact proteins and peptides in the gastrointestinal tract as a protective mechanism contributing to gut-associated-lymphoid-tissue (GALT) education and microbiota shaping (Chatterton et al., 2013; Newburg & Walker, 2007). Thus, the improved proteolysis of PHM compared to RHM is not necessarily advantageous.

The impact of Holder pasteurization on the lipolysis and proteolysis kinetics observed here for the preterm milk was somewhat lower than that observed for term milk (de Oliveira et al., 2015). The pre-lipolysis levels in RHM and PHM were lower in preterm compared to term human milk. The difference in prelipolysis between RHM and PHM preterm milk was also lower than in term milk. This initial pre-lipolysis level seems to impact further the kinetics of hydrolysis, which partially explains the lower

impact of pasteurization observed during preterm newborn digestion. Indeed, the occurrence of lipolysis and proteolysis during storage and processing of human milk was already demonstrated and discussed by several authors (Dallas et al., 2015; de Oliveira et al., 2015; Borgo et al., 2014; Armaforte et al., 2010), and is linked to the action of endogenous enzymes of human milk associated to freeze-thaw cycles and pasteurization. Furthermore, three factors may explain the lower hydrolysis degrees during preterm compared to term newborn digestion: firstly, higher protein and lipid contents in preterm milk; then, reduced time of passage of the meal in the gastric compartment in preterm associated with higher pH levels; thirdly, lower proportion of enzymes and bile compared to residual meal during preterm gastrointestinal digestion.

The *in vitro* dynamic system used in our study to simulate preterm newborn digestion is a pertinent tool but have some limitations. For instance, this model does not simulate absorption, hormonal feed-backs and the role of microbiota on digestion. An *in vivo* study is currently being carried out by our laboratory in order to evaluate the impact of pasteurization on mother's own fresh human milk gastric digestion in hospitalized preterm newborns (NCT02112331).

2.2.6. Conclusion

In conclusion, Holder pasteurization impacted the disintegration patterns and the digestion of some human milk components with recognized nutritional relevance (AA and FA). This may have some physiological repercussion. Furthermore, some nutrients not analyzed in our study may suffer big losses after pasteurization. The insufficient ingestion and assimilation of these components may limit the early preterm healthy development and growth, since they are linked to beneficial short-term effects such as preventing infection and inflammation or promoting establishment of beneficial microbiota and intestinal maturity. However, to help the preterm newborn to overcome the critical period, the pasteurized human milk is still preferred against infant formula. It is important to monitor the recommended intake of nutrients, especially for preterm infants receiving pasteurized donor milk (often coming from mothers of term infants). Adequate fortification of this milk is thus often recommended. Our results reinforce the necessity of carefully control and optimize all the steps in the human milk processing (from expression to distribution) in order to minimize the nutrient losses and modifications, including to explore alternative treatments to Holder pasteurization.

Acknowledgements: The authors warmly thank the volunteer mothers for their donations of breast milk samples and the colleagues from the bank milk of the Rennes University Hospital Center for their involvement in the project. This work was integrated in the COST action FA1005 INFOGEST, and the researchers associated are acknowledged for contribution to the discussion on digestion parameters.

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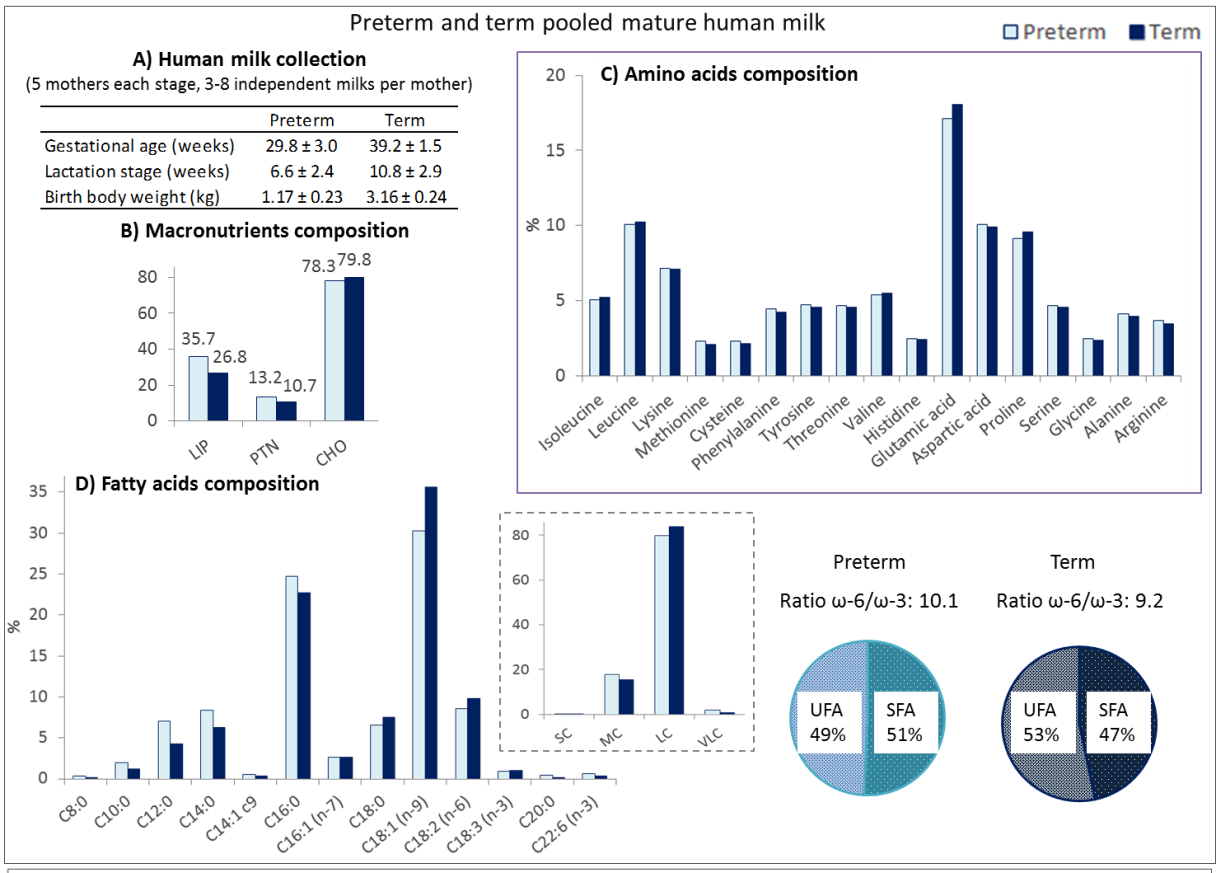
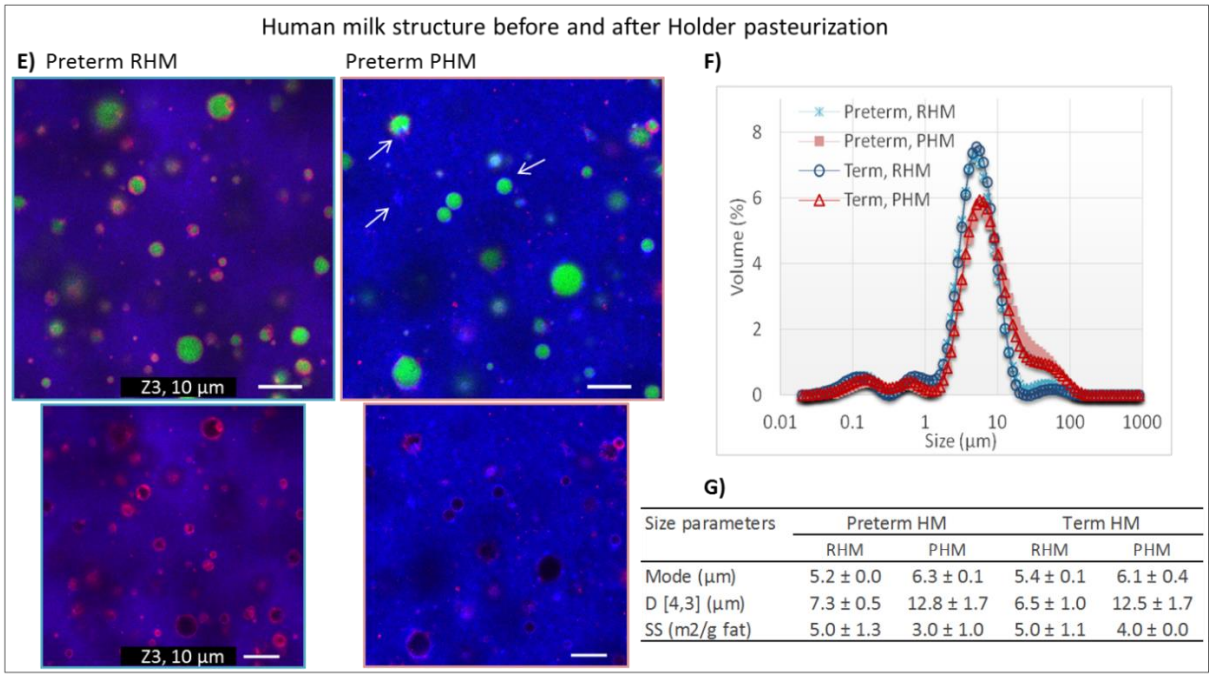
2.2.7. Supplementary material

Supplementary Table 1. Amino acids composition of undigested preterm human milk

Amino acid (g/kg of milk)	Total amino acids	Free amino acids
Isoleucine	0.523	0.000
Leucine	1.045	0.006
Lysine	0.744	0.002
Methionine	0.242	0.000
Cysteine	0.241	0.004
Phenylalanine	0.461	0.000
Tyrosine	0.489	0.002
Threonine	0.487	0.009
Valine	0.560	0.004
Histidine	0.255	0.004
Glutamic acid	1.772	0.280
Aspartic acid	1.041	0.010
Proline	0.949	0.002
Serine	0.486	0.008
Glycine	0.258	0.006
Alanine	0.427	0.021
Arginine	0.383	0.000
Taurine	-	0.035
Amino nitrogen	1.328	0.042
Total nitrogen	2.064	

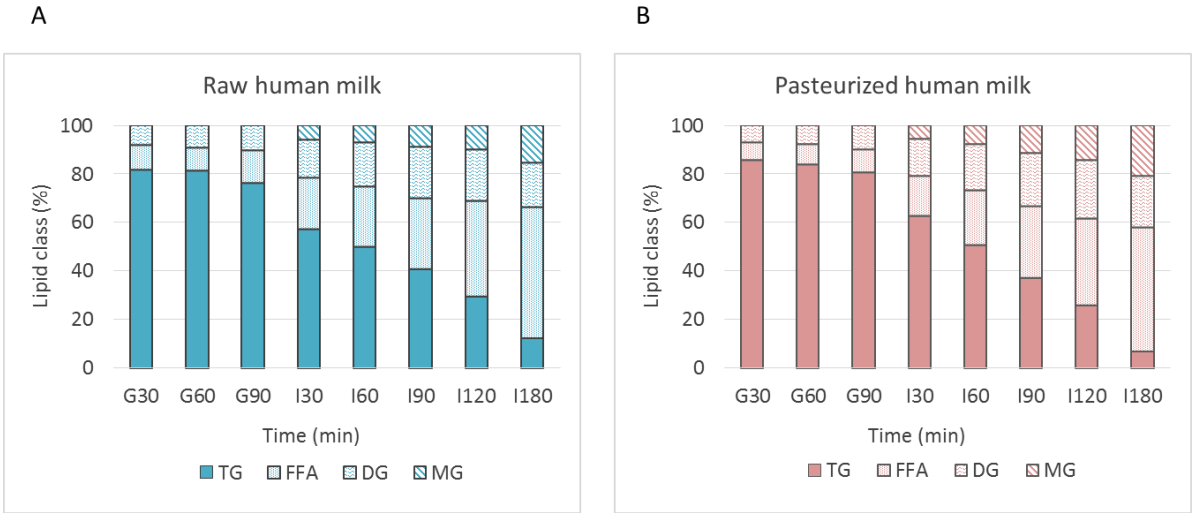


	Preterm	Term
Gestational age (weeks)	29.8 ± 3.0	39.2 ± 1.5
Lactation stage (weeks)	6.6 ± 2.4	10.8 ± 2.9
Birth body weight (kg)	1.17 ± 0.23	3.16 ± 0.24

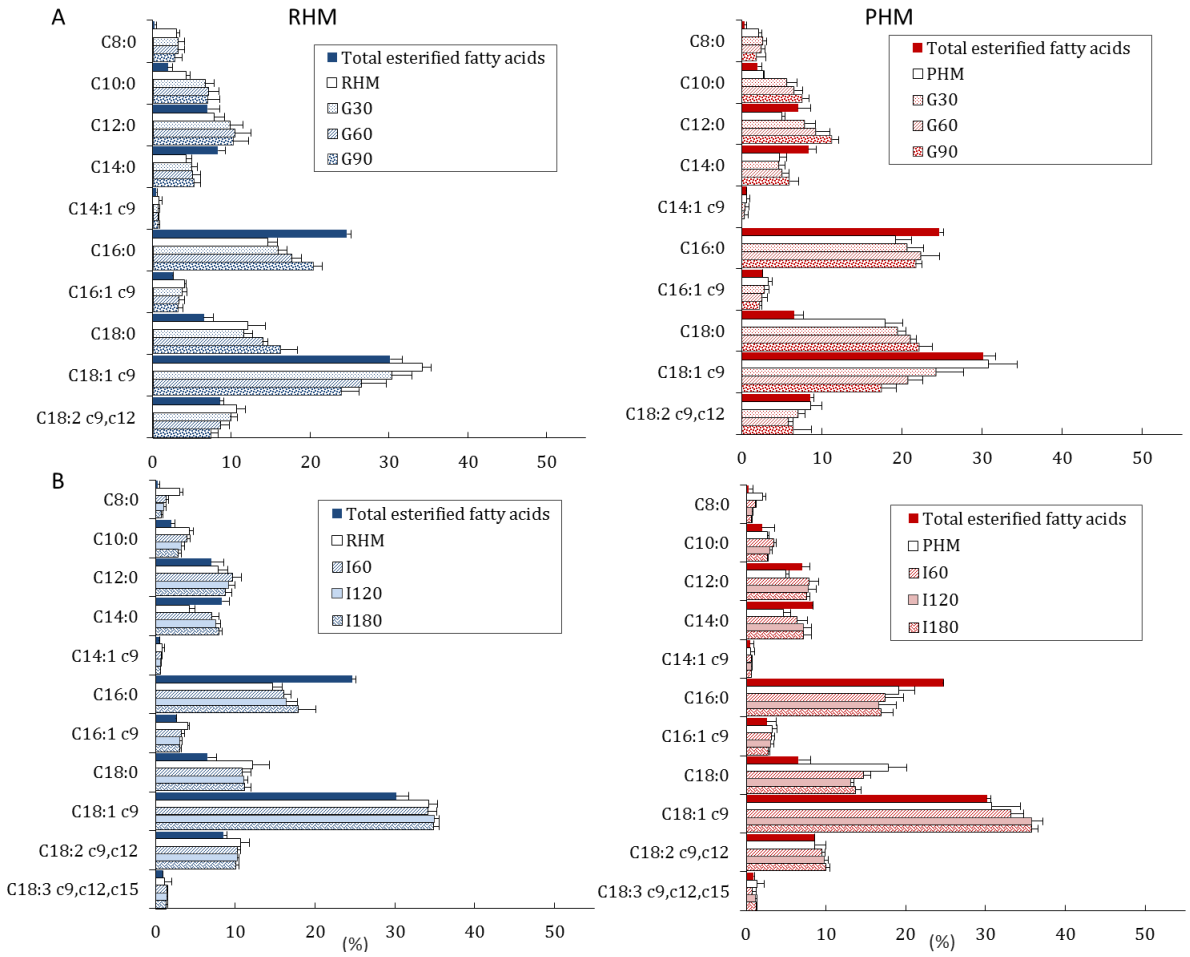


Size parameters	Preterm HM		Term HM	
	RHM	PHM	RHM	PHM
Mode (µm)	5.2 ± 0.0	6.3 ± 0.1	5.4 ± 0.1	6.1 ± 0.4
D [4,3] (µm)	7.3 ± 0.5	12.8 ± 1.7	6.5 ± 1.0	12.5 ± 1.7
SS (m2/g fat)	5.0 ± 1.3	3.0 ± 1.0	5.0 ± 1.1	4.0 ± 0.0

Supplementary Figure 1. Comparison of preterm and term undigested mature human milk: A) human milk collection conditions before pool; B) macronutrients, C) amino acids and D) fatty acids composition of pooled human milk. Pooled human milk structure before and after Holder pasteurization: E) confocal laser scanning microscopy images (x60 zoom 3) of preterm human milk before and after pasteurization (white narrows are pointing at heat-induced protein aggregation; proteins are colored in blue (FastGreen®), apolar lipids in green (Lipidtox®) and polar lipids in red (Rhodamine-PE®); F) particle size distribution and G) associated size parameters of preterm and term human milk, before and after pasteurization (as determined by laser light scattering). For A) and G) data point represents means ± SD. LC, long-chain; MC medium-chain; PHM, pasteurized human milk; RHM, raw human milk; SC, short-chain; SFA, saturated fatty acids; SS, specific surface area; UFA, unsaturated fatty acids; VLC, very long chain.



Supplementary Figure 2. Evolution of the lipid classes composition (acyl chains, mol %) of preterm raw (A) and pasteurized human milk (B) during the gastrointestinal *in vitro* dynamic preterm digestion as determined by thin layer chromatography coupled to a flame ionization detector. G, gastric phase followed by time in min; I, intestinal phase followed by time in min. TG, FFA, DG and MG are respectively triglycerides, free fatty acids, diglycerides and monoglycerides.



Supplementary Figure 3. Acyl chains profiles initially esterified in preterm human milk compared to free fatty acids released from raw (RHM) and pasteurized human milk (PHM) during gastric (A) and intestinal (B) *in vitro* dynamic preterm digestion. Fatty acids were determined by gas chromatography. Data point represents means (n=3 for each milk) ± SD of mass percentage. G, gastric phase followed by time in min; I, intestinal phase followed by time in min.

2.3. Is the impact of pasteurization on HM digestion different in term and preterm newborns?

The results presented in sections 2.1 and 2.2 confirmed the impact of Holder pasteurization on the HM digestion. However, this impact seemed less pronounced in preterm compared to term stage, although a direct comparison of data was not undertaken. In brief, during gastric digestion pasteurization slowed down the lipolysis degree of term HM, while for preterm HM this impact was limited to the bioaccessibility of some FA; proteolysis was slowed down for β-casein and accelerated for lactoferrin in term HM, while no impact was observed for preterm HM proteins. During the intestinal phase, lipolysis degree was lower after pasteurization in term and preterm stages, but with a smaller extent of difference in the later. Overall, preterm HM had lower hydrolysis levels than term HM.

As briefly discussed in section 2.2, differences in the digestion behavior of preterm and term HM were expected due to differences in their composition (**Supplementary Figure 1**, section 2.2) and in the digestion parameters specific of each stage (*i.e.* lower enzymes and bile salts levels and faster gastric emptying rate in the preterm, **Table 4** in section 2.1 and **Table 7** in section 2.2).

Preterm HM being less hydrolyzed than term HM is not surprising. Indeed, preterm HM emptied faster than term HM from the gastric into the intestinal compartment, the proportion of residual preterm HM contributing to the total volume was lower in the gastric phase and higher in the intestinal phase. In addition, at any time of digestion the ratio between enzyme activity and the meal was higher for term compared to preterm digestion. These differences are illustrated in **Figure 22**.

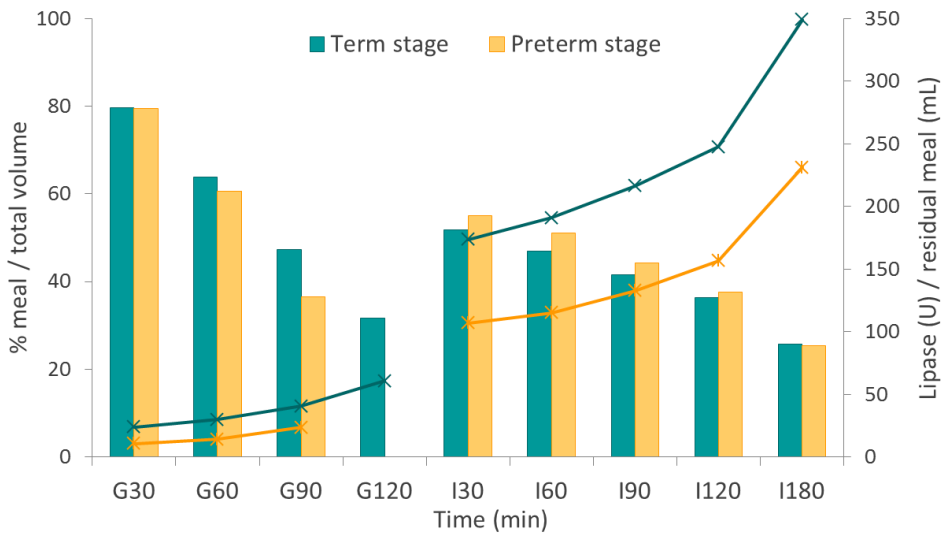


Figure 22. Contribution of residual meal to the total gastric and intestinal contents after ingestion of term and preterm HM (histograms) and the corresponding ratio between enzyme activity and residual meal (lines). G and I stand for gastric and intestinal phases respectively, followed by time in min.

However, in addition to these stage-dependent differences in digestive conditions, it is important to underline that undigested term HM presented considerable difference in pre-lipolysis degrees between RHM and PHM (5%), which was maintained during the gastric and the intestinal digestion. On the contrary, undigested preterm RHM and PHM presented no difference between their pre-lipolysis degrees, which was maintained during gastric digestion; during the intestinal digestion, the presence of BSSL in the RHM may have contributed to increase its lipolysis degree as compared to PHM. Considering that from 26th weeks of GA the mammary gland synthetizes BSSL, and that its levels are the same in preterm and term HM, a different activity does not explain this difference of pre-lipolysis levels between term and preterm RHM (Freed et al., 1987). However, experimental constraints may explain the higher pre-lipolysis degree observed for term RHM, as this milk was stored for a longer time between collection and thawing for pooling (on average 93 days against 43 days for preterm HM). This storage period was linked to the length of time necessary to recruit donors by HMB and to the frequent advanced stage of lactation mainly concerning donors delivering at term. Remarkably, this length of storage remained shorter than the longest period allowed by HMB (four months maximum at -20 °C for RHM).

It is important to underline that pre-lipolysis can occur before the freeze-thaw cycle applied in our scheme of milk storage and also in HMB before pasteurization. Another limit in our approach, which may have enhanced the difference between PHM and RHM, is that before each digestion RHM underwent a second freeze-thaw cycle. This second cycle very likely facilitated the action of endogenous lipolysis and did not correspond to the real practice. To avoid this bias, it would have been necessary to carry out the three digestions of RHM within the 24h following the first cycle of freeze-thaw and pool. One option could have been, for example, to have miniaturized *in vitro* digestion systems in series. In our case, such equipment was not available and was impossible to build during the timescale of the project; therefore, we have chosen the methodology which allowed to perform triplicate digestions of the same pool of milk, in order to obtain pools of RHM or PHM having strictly the same chemical compositions, and differing only in terms of processing.

In summary, pasteurization impact was higher in the term stage notably due to the higher pre-lipolysis levels in term RHM compared to term PHM. However, we have not enough elements to generalize and make sure this difference would be always observed in HMB processing chain. It would be interesting to investigate the link between the pre-lipolysis level and the BSSL activity or bile salts concentration in HM. Finally, the impact of pasteurization on HM digestion needs to be confirmed *in vivo*.

Chapter 3 – Impact of human milk pasteurization on gastric digestion in preterm infants: a randomized controlled trial

Based on the following publication:

The American Journal of Clinical Nutrition
Official publication of the American Society for Nutrition

Impact of human milk pasteurization on gastric digestion in preterm infants: a randomized controlled trial[1,2]

Samira C de Oliveira,[3] Amandine Bellanger,[4,5] Olivia Ménard,[3] Patrick Pladys,[4,5] Yann Le Gouar,[3] Emelyne Dirson,[4] Florian Kroell,[3] Didier Dupont,[3] Amélie Deglaire,[3] and Claire Bourlieu[3]*

[3]Science and Technology of Milk and Eggs, Agrocampus West, French National Institute for Agricultural Research, Rennes, France; [4]Department of Pediatrics, Rennes University Hospital, Rennes, France; and [5]Faculty of Medicine, University of Rennes 1, Rennes, France

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In chapter 2 we showed that pasteurization impacted the *in vitro* digestion of term HM and, in a lesser extent, of preterm HM. Although carefully adapted to the stage of the newborn and to the meal digested, **the *in vitro* digestion system does not reproduce the biological complexity of the digestive tract (*i.e.* absorption, hormonal feedbacks, neural interactions, peristaltic movements).** Furthermore, *in vitro* parameters, such as half-time of gastric emptying, were similar for PHM and RHM, while differences may occur but have not been investigated in previous studies. *In vivo* studies, particularly in infants, are rarely performed due to obvious ethical constraints and technical difficulties.

Thus, **it appears important to provide information about the behavior of RHM and PHM during *in vivo* digestion, and to confirm or not the impact of pasteurization on this behavior**. In this context, the main goal of this chapter was to answer the following question:

Does Holder pasteurization impact, in vivo, the gastric digestion of HM in preterm infants fed their mothers' own milk?

This *in vivo* study was part of a clinical trial named ARCHILACT, which was planned and performed in collaboration with the University Hospital Center of Rennes (Clinical Trials NCT02112331). Due to ethical constraints, it concerned only the gastric phase of digestion, accessible through the nasogastric feeding tube of the preterm newborns, which was not implemented on purpose of our study. Preterm hospitalized tube-fed newborns were included in one out of two completely independent groups: in group A when mothers were able to give their own milk; in group B when HM from anonymous donor was necessary. Mothers were not influenced in their decision of giving their own milk or not.

Likewise the *in vitro* study, samples were withdrawn at different times during the gastric digestion and analyzes were performed in order to assess the kinetics of lipolysis, of proteolysis and the emulsion disintegration of the different types of milk. Gastric volume, pH and gastric emptying rate were described.

The present study was published in the American Journal of Clinical Nutrition.

3.1. Abstract

Background: Holder pasteurization has been reported to modify human milk composition and structure, by inactivating bile-salt stimulated lipase (BSSL) and partially denaturing some of its proteins, potentially affecting its subsequent digestion.

Objective: We sought to determine the impact of human milk pasteurization on gastric digestion (particularly for proteins and lipids) in preterm infants who were fed their mothers' own milk either raw or pasteurized.

Design: In a randomized controlled trial, 12 hospitalized tube-fed preterm infants were their own control group in comparing the gastric digestion of raw human milk (RHM) with pasteurized human milk (PHM). Over a 6-d sequence, gastric aspirates were collected 2 times/d before and after RHM or PHM ingestion. The impact of milk pasteurization digestive kinetics and disintegration was tested with the use of a general linear mixed model.

Results: Despite inactivating BSSL, instantaneous lipolysis was not affected by pasteurization (mean ± SD at 90 min: 12.6% ± 4.7%; $P > 0.05$). Lipolysis occurred in milk before digestion and was higher for PHM than for RHM (mean ± SD: 3.2% ± 0.6% and 2.2% ± 0.8%, respectively; $P < 0.001$). Pasteurization enhanced the proteolysis of lactoferrin ($P < 0.01$) and reduced that of α-lactalbumin (only at 90 min) ($P < 0.05$). Strong emulsion destabilization was observed, with smaller aggregates and a higher specific surface for PHM ($P < 0.05$). Pasteurization did not affect gastric emptying (~30-min half time) or pH (mean ± SD: 4.4 ± 0.8 at 90 min).

Conclusions: Overall, pasteurization had no impact on the gastric digestion of lipids and some proteins from human milk but did affect lactoferrin and α-lactalbumin proteolysis and emulsion disintegration. Freeze-thawing and pasteurization increased the milk lipolysis before digestion but did not affect gastric lipolysis. Possible consequences on intestinal digestion and associated nutritional outcomes were not considered in this study.

This trial was registered at clinicaltrials.gov as NCT02112331

Keywords: Digestion, Holder pasteurization, human milk, infant nutrition, preterm infant.

3.2. Introduction

The nutritional management of preterm infants plays an essential role in recovering their limited intrauterine fetal growth and development and thus in minimizing subsequent negative outcomes. Mothers' milk (rather than infant formula) is recommended for preterm infants because its administration improves preterm outcomes, with reported positive impacts in terms of gastrointestinal tract maturation, tolerance to feeding, and the prevention of inflammation and

infection risks (Moro et al., 2015; Quigley & McGuire, 2014; Rozé et al., 2012; Schanler et al., 2005). Because most infants do not develop the suck-swallow-respiration coordination before 36 to 38 weeks of gestation, enteral nutrition through a nasogastric tube is necessary (Rayyan et al., 2015). For enteral administration, mothers usually collect their breast milk, which will be either refrigerated if used between 24 and 48 h or frozen at -20 °C and sent to the human milk bank (HMB) for storage and possibly processing (as occurs at our institution) (Picaud, 2015). To ensure the sanitary quality of the human milk, Holder pasteurization (62.5°C, 30 min) is recommended by the worldwide guidelines (HMBANA, 2015; Arslanoglu et al., 2010; Hartmann et al., 2007). During this process, 2 cycles of freeze-thawing are applied: one after milk expression before pasteurization and another after pasteurization before administration (Borgo et al., 2014). When milk from their own mother is not available, infants can receive milk from an anonymous donor that undergoes the same steps of storage and processing in the HMB.

Various authors have demonstrated an impact of processing and pasteurizing human milk on its composition (O'Connor et al., 2015; Akinbi et al., 2010), microstructure (de Oliveira et al., 2016; Lopez et al., 2013), hydrolysis by endogenous enzymes of milk (de Oliveira et al., 2015; Bertino et al., 2013; Bitman et al., 1983), and physiologic and nutritional aspects (Andersson et al., 2007; Thomaz et al., 1999; Williamson et al., 1978). Despite these impacts, beneficial health outcomes have been reported in preterm infants fed with donors' pasteurized human milk (PHM) compared with infant formula, especially those related to necrotizing enterocolitis (de Halleux et al., 2016; Quigley & McGuire, 2014). Among such modifications on milk, some may negatively affect the ability of infants to digest lipids and proteins because of i) the total inactivation of endogenous human milk lipases (bile-salt stimulated lipase, BSSL, and lipoprotein lipase) (Henderson et al., 1998); ii) the potential activation of the endogenous plasmin system (Deglaire et al., 2016); or iii) the heat-induced protein denaturation and aggregation on the milk fat globule membrane (MFG). This last factor is less often taken into account but could modulate the enzymes' ability to hydrolyze (Bourlieu et al., 2015a; de Oliveira et al., 2015; Raikos, 2010).

In vitro studies conducted in a dynamic gastrointestinal model have recently reported that the pasteurization of human milk affects its kinetics of hydrolysis and disintegration in term newborns (Deglaire et al., 2016; de Oliveira et al., 2015). The impact was less in preterm newborns, but it still affected disintegration, intestinal lipolysis, and the bioaccessibility of some fatty acids (FAs) and amino acids (de Oliveira et al., 2016). Although this dynamic model was validated as a relevant tool for studying infant digestion (Ménard et al., 2014), it did not include certain *in vivo* conditions such as the feedback mechanisms of digestive hormones, neural interactions, and absorption. In this context, we sought to investigate the impact of human milk pasteurization on subsequent gastric digestion in

preterm infants fed with their mothers' milk. More specifically, this impact was determined in terms of lipolysis, proteolysis, and structural disintegration.

3.3. Methods

Study design and participants

This study (NCT02112331) was a randomized controlled trial conducted from April 2014 to August 2015 at Rennes University Hospital. It was approved by the Nantes University Institutional Review Board.

Figure 23 shows the flow diagram of the infants included in the study. Hospitalized preterm newborns were assessed for eligibility according to the following criteria: 1) gestational age at birth <32 wk, 2) a postpartum hospital stay, 3) nasogastric tube feeding, and 4) the mother's intention to breastfeed during the hospital stay. Infants were then pre-included within 72 h from birth after their parents received oral and written information by the pediatricians and gave informed written consent. Once an infant reached an enteral feeding ≥120 mL/kg/d at 3-h intervals and the mother had expressed sufficient milk for 6 meals (pasteurized modality), a medical visit was performed to assess the infant for inclusion in the study. Exclusion criteria were 1) any substantial digestive disease or malformation, including a previous history of necrotizing enterocolitis; 2) abdominal bloating with abdominal tension during the experimental protocol; 3) treatment with catecholamine or morphine during the experimental protocol; 4) sampling failure (volume <4 mL) on 2 samples taken at the same postprandial time point (except 90 min) and for the same type of milk.

Each infant was his or her own control in comparing the gastric digestion of raw human milk (RHM) with PHM. A power analysis study showed that a sample size of 12 infants was needed to detect a significant difference in the gastric lipolysis degree (LD) between RHM and PHM, with 80% power at the significance level of 0.05. Because no study to our knowledge has directly compared the gastric lipolysis of RHM with PHM, the study of Armand et al. (1996) in preterm infants, which detected a reduction of ~40% in the lipolysis level after the ingestion of infant formula compared with RHM, served as a basis for the power analysis.

Test meals

<u>RHM</u>

RHM was collected from each infant's own mother at the hospital ≤24 h before administration and was stored at 4 °C in the neonatology unit.

<u>PHM</u>

At least 3 d after the beginning of the lactation, the infant's own mother started expressing her milk for collection (at the hospital or at home) following the usual service instructions. This milk was frozen at -20 °C within 24 h and then transferred to the HMB in cooled boxes within 1 h. Milk samples from the same mother were thawed in a 4 °C temperature-controlled room for ≤24 h, pooled, divided into 6 bottles (60–75 mL) for each of the 6 test meals, and underwent Holder pasteurization (62.5 °C for 30 min) before being returned to storage at -20 °C. Bacteriologic tests were performed on 2 samples from each pool — one taken after pooling the milk and another immediately after pasteurization. Milk was considered adequate for consumption if the total aerobic bacterial load at 37 °C was <1 X 10^6 CFU/mL and coagulase-positive Staphylococci were <1 X 10^4 CFU/mL for the pooled milk before pasteurization; total aerobic bacterial load at 37 °C was <2 CFU/mL for the milk after pasteurization. Before each administration, a bottle of PHM was thawed at 4 °C for ≤24 h.

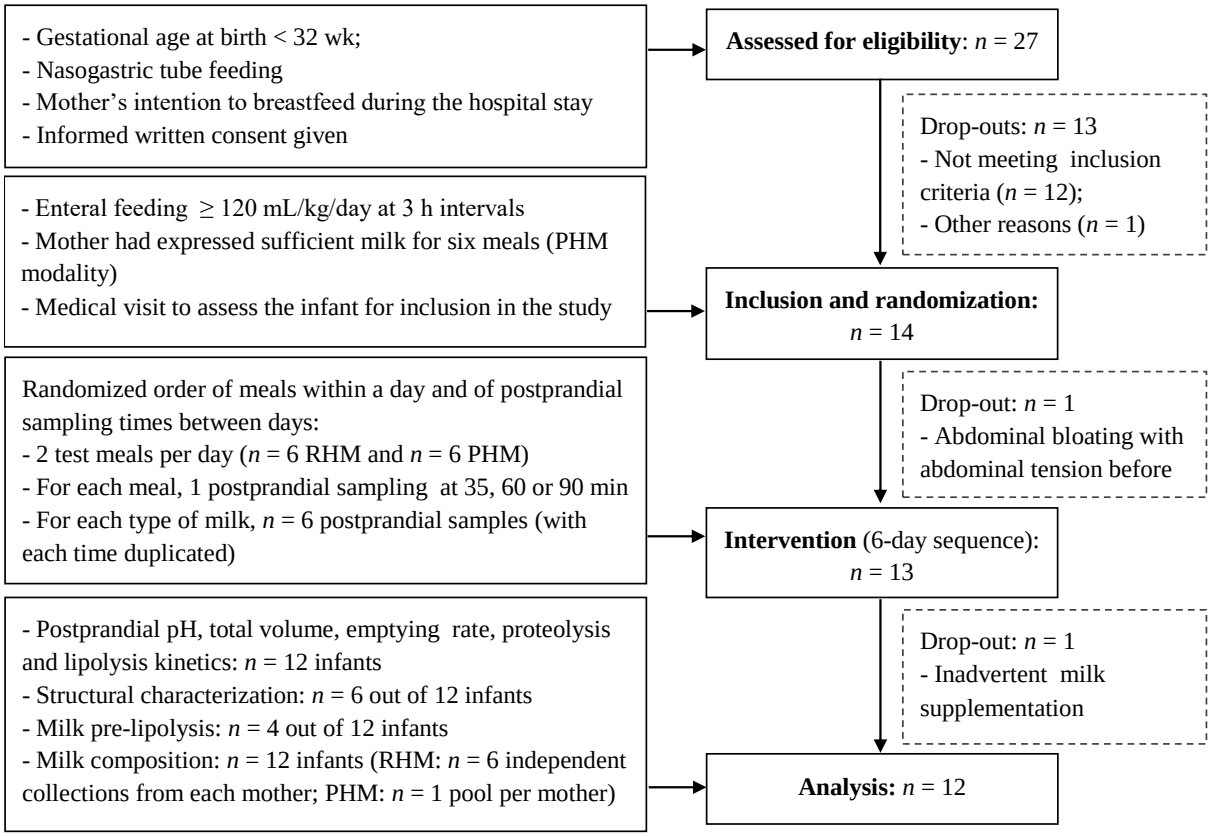


Figure 23. Flow diagram of infants included in the study. The number of samples potentially collected per modality is indicated. Only 1 group was formed: each infant was his or her own control in comparing the gastric digestion of PHM and RHM. PHM, pasteurized human milk; RHM, raw human milk.

Experimental protocol

The experimental protocol was applied over a 6-d sequence, with both test meals (RHM and PHM) administered each day at 08:30 am or 02:30 pm. These 2 test meals out of the 8 daily feeds were not

supplemented. The total gastric content was aspirated through the nasogastric tube, first before the test meal feeding (fasting state) and again at a given time after the beginning of the feeding (35, 60, or 90 min). Each postprandial time collection was duplicated during the 6-d sequence. The orders of the meal administration within a day and of the postprandial collection time between days were randomly determined, but with the condition that all 3 postprandial times should be used during the first 3 d of the sequence. The randomization tables were previously prepared with the use of a computerized randomization tool and chosen by a computerized draw of lots 1 d before the beginning of the 6-d period.

The amount of test meals was fixed at 20 mL/kg of body weight and administrated by a feed pump for 30 min. Before each administration, a 6-mL milk sample was taken for subsequent analyses for each of the 6 d for the RHM (when possible) but just 1 time/wk, in duplicate, for the PHM because all PHM feeds came from the same pool of milk (**Figure 23**). The pre-feed content (fasting state) was measured by totally aspirating the gastric content with the use of a 5–10-mL syringe with 0.5 mL graduation; this aspirated content was then given back to the infant via the nasogastric tube at a maximum volume of 2 mL to avoid any influence of the previous meal and to standardize the amount of fasting secretions within each infant. At each postprandial collection, the total volume was collected, and its amount was recorded; 4–6 mL digesta were then aliquoted into sterile tubes for pH measurements and subsequent analyses, and the unused digesta were then immediately given back to the infant with the use of a syringe by gravity feeding. On any occasion when the aspirated volume was ≤4 mL, all of the removed digesta were given back (except at 90 min), and the sampling point was then considered as a failure. After each successful aspiration of the gastric contents, the digesta were gently mixed with the use of the syringe to ensure homogeneity before sampling and return of the surplus. To compensate for the digesta removed for sampling, the infants received an additional 2 mL milk/kg on each of the 6 other normal daily feeds.

Handling of samples

The volume and pH of gastric content samples were measured immediately after collection, as was the pH of the milk samples. The macronutrient composition of RHM and PHM was measured from a 2-mL sample that had been stored at -20 °C before analyses. Analysis was by infrared spectrophotometry with the use of a Human Milk Analyzer (Miris AB) calibrated with reference methods (Billard et al., 2015).

All samples used to determine structural characterization were kept at 4 °C and analyzed within 4 h. Aliquots taken for milk macronutrients and total FA composition analyses were immediately frozen (-20 °C). Other aliquots were either submitted to direct Folch extraction or frozen after the addition of a protease inhibitor, *i.e.*, 10-µL solution of 0.72 M Pepstatin A (Sigma-Aldrich) per milliliter of sample.

The method for the Folch extraction is briefly described as follows: 400 µL of sample mixed with 2.4 mL chloroform:methanol (2:1 *v/v*) and acidified with 160 µL 0.1 N HCl. To ensure the purity of the lipids, the extract was mixed with 96 µL 0.73% sodium chloride and 600 µL chloroform:methanol (2:1 *v/v*). The chloroform phase was recovered for further lipid analyses.

Analytic methods

Structural characterization

The microstructure of human milk and digesta was observed using a confocal laser scanning microscopy (CLSM) on inverted microscope (TE2000-E; Nikon) and laser light scattering with 2 laser sources (Mastersizer 2000; Malvern Instruments), as previously described by Bourlieu et al. (2015a). From the particle size measurements, the mode diameter, the surface weighted mean (D [3, 2]), the volume weighted mean (D [4,3]) and the specific surface area of MFG were calculated.

Structural characterization analyses were performed for the first 6 infants of the 12 included in this study. For each of these infants, 1 sample of the corresponding pool of PHM and 2 samples of the 6 RHM meals were randomly selected and analyzed; postprandial gastric digesta were analyzed once for each type of milk (RHM or PHM) and for each time point (35, 60, and 90 min).

Lipid analyses

Three complementary lipid analyses were conducted on the milk and digesta samples: i) analysis of lipid classes (residual triglycerides, diglycerides, monoglycerides and free FA) by thin layer chromatography coupled to a flame ionization detector (IATROSCAN MK5 equipment, Iatron Laboratories); ii) analysis of free FA (C8:0 to C18:3) by gas chromatography after solid-phase extraction; iii) total esterified FA (C8:0 to C24:0) by gas chromatography coupled to a flame ionization detector. The first two analyzes were performed on the chloroform phase recovered after direct Folch extraction, and the last one after direct transmethylation. These analyses were previously described by de Oliveira et al. (2015).

Protein analyses

SDS-PAGE analyses were performed with the use of 4–12% polyacrylamide NuPAGE® Novex® Bis-Tris 15-well precast gels (Invitrogen), following the manufacturer's instructions. All samples were diluted 4-fold with NuPAGE® LDS sample buffer and then treated with 0.5 M DL-dithiothreitol and distilled water. A total of 3–10 µg protein was deposed per gel (based on the milk protein composition), and each sample was deposed twice. Mark 12 Unstained Standard (Invitrogen) was used as a molecular weight marker. Gels were fixed in 30% (*v/v*) ethanol, 10% (*v/v*) acetic acid and 60% (*v/v*) deionized water and then rinsed for 15 min in deionized water before staining with Coomassie Blue. Image analysis of SDS-PAGE gels was carried out using Image Scanner III and LabScan 6.0 (GE Healthcare).

After digitization of the gel images, bands were selected and their grey intensity determined by densitometry with the use of Image Quant TL (GE Healthcare). Densitometry analyses were performed for semi-quantification of intact proteins.

Free taurine content (used as a meal dilution marker) was determined after the samples were deproteinized with the use of sulfosalicylic acid (Sigma Aldrich) as previously described by de Oliveira et al. (2015). Briefly, milk and digesta aliquots (1 mL) were treated with 50 mg of sulfosalicylic acid and incubated for 1 h at 4 °C. The mixtures were centrifuged at 5000 × g for 15 min at 4 °C, and the supernatant was filtered through a 0.45 µm pore-size membrane (Sartorius). The filtrate was diluted five times with a 0.2 M lithium citrate buffer (pH 2.2) before injection. The analyses were carried out with the use of cation exchange chromatography on a Biochrom 30 automatic amino acid analyzer (Biochrom Ltd.), with the use of lithium citrate buffers as eluant and ninhydrin post-column reaction system.

Calculations

The instantaneous *LD* (percentage), *i.e.,* the concentration of FFAs compared with total FAs present in the digesta at a given time, was calculated as follows:

$$LD = \frac{100 \times FFA}{(3 \times TG + 2 \times DG + MG + FFA)} \quad (1)$$

where *FFA*, *TG*, *DG*, and *MG* concentrations are expressed as mol/L.

The global *LD* (percentage) between 35 and 90 min was calculated from the total amount of FAs released during this period compared with the total FAs initially esterified in the milk. The proportion of each FA released from RHM and PHM after 90 min of digestion (% *wt:wt*) was calculated as follows:

$$\text{FA released} = \left(\frac{[FFA_{90}] \times Vol_{90}}{[TFA_{meal}] \times Vol_{meal}}\right) \times 100 \quad (2)$$

where $[FFA_{90}]$ and $[TFA_{meal}]$ are, respectively, the concentration of FFAs (mg/mL) in the stomach at 90 min and the total FAs in the meal ingested. Vol_{90} and Vol_{meal} are, respectively, the total gastric volume aspirated at 90 min and the corresponding volume of milk ingested (mL).

The percentage of each intact protein remaining in the stomach at a given time was estimated in comparison with undigested human milk with the use of the following equation:

$$\text{\% of intact protein} = 100 \times \left(\frac{\text{Protein } GI_{digesta}}{\text{Protein } GI_{meal}}\right)$$

(3)

where *Protein GI* is the grey-band intensity of each protein obtained by densitometry analysis.

The percentage of residual meal (*RM*) remaining in the gastric contents at a given time was estimated with the use of the amino acid taurine as a meal marker in the soluble phase. Taurine is present in human milk only in the free form; it is neither produced nor absorbed during gastric digestion. The percentage of *RM* was thus calculated according to the following equation:

$$RM = \left(\frac{[Tau_{digesta}]}{[Tau_{meal}]}\right) \times 100$$

(4)

where $[Tau_{digesta}]$ and $[Tau_{meal}]$ are the taurine marker concentrations (mg/mL) present in the gastric content and in the meal.

The volume of meal present in the stomach (mL) was derived from RM by multiplying it by the corresponding total gastric volume at the corresponding postprandial time. The difference between the total gastric volume and the volume of meal consumed (mL) gave an estimation of the volume of secretion in the gastric content by this time.

The gastric emptying rate (*GE*) of milk, defined as the percentage of the ingested meal remaining in the stomach at a given time, was calculated as follows:

$$GE = \left(\frac{[Tau_{digesta}] \times Vol_{digesta}}{[Tau_{meal}] \times Vol_{meal}}\right) \times 100$$

(5)

where $[Tau_{digesta}]$ and $[Tau_{meal}]$ are the taurine marker concentrations (mg/mL) in the gastric contents and in the meal. $Vol_{digesta}$ and Vol_{meal} are the volumes (mL) of the gastric contents and milk ingested, respectively.

During the meal ingestion (30 min) and five min thereafter, no samples were withdrawn. The gastric volume for this period was thus estimated assuming that i) the gastric emptying process started five min after the beginning of feeding and ii) the rate of emptying was constant from 5 to 35 min, thus:

$$\text{Estimated Vol} = (Meal_t + Secretion_t) - \left[\left(\frac{Meal_{ing} + Secretion_{35} - Vol_{35}}{30}\right) \times (t-5)\right]$$

(6)

where *Estimated Vol* is the estimated gastric volume (mL/kg) at *t* (time in minutes after the beginning of the feeding). $Meal_t$ and $Secretion_t$ are, respectively, the milk ingested and secretion produced at *t*

(mL/kg). $Meal_{ing}$ is the total milk ingested (20 mL/kg), $Secretion_{35}$ is the mean secretion produced considering values at 35 min (mL/kg), and Vol_{35} is the mean total gastric volume at 35 min (mL/kg).

The difference between the estimated gastric volume (*Estimated Vol)* and the secretion at a given time *t* ($Secretion_t$) was considered as the RM in the stomach (mL/kg). The RM (mL/kg) at a given time ($Meal_t$) was used to obtain the percentage fraction of the ingested meal remaining in the stomach *(Estimated GE)* for a total of 20 mL meal/kg as follows:

$$\text{Estimated GE} = \frac{\text{Meal}_t}{20} \times 100 \tag{7}$$

Statistical analyses

Statistical analyses were conducted with the use of R version 3.1.2 (R Core Team, 2014). A paired t test was conducted to assess the impact of pasteurization on human milk (before administration) and on the FA release from RHM and PHM after 90 min of digestion. The variables concerning human milk before use were particle size parameters, degree of prelipolysis (lipolysis of milk before digestion triggered by its endogenous enzymes), macronutrient content, and FA proportion.

The impact of pasteurization on the subsequent digestive kinetics was tested on the response variables (*Y*) with the use of a linear mixed model after a model selection starting from the initial model:

$$Y = \text{Meal} + \text{Time} + \text{Meal x Time} + \text{Subject} + \textit{Sampling day} + \textit{Body weight} + \textit{Lipid content} + \textit{Protein content} \tag{8}$$

Subject was considered as random effect, and other parameters were fixed effects. *Body weight* corresponds to the subject's body weight (kg) at each sampling day (day 1 to 6), and *Lipid* and *Protein contents* refer to the composition of the ingested milk and were excluded from the models for response variables concerning either proteolysis or lipolysis, respectively. The model selection was conducted on the initial model among the covariates *Body weight*, *Sampling day*, *Lipid content* and/or *Protein content* to choose the most parsimonious model. This choice was based on the Akaike information criterion (smaller is better) with the use of the "stepAIC" function from the "MASS" package. From the selected model, an analysis of deviance (function "anova.lme" from the package "nlme") was carried out with the use of the Wald chi-square test to determine the p-values for *Meal*, *Time* and *Meal x Time*. When differences were significant (p-value < 0.05), post hoc tests were performed using a Bonferroni correction (function "glht" from the package "multcomp"). The distribution normality of the residuals from the selected linear mixed models was tested for each

response variable with the use of the Kolmogorov-Smirnov test (function "lillie.test" from the package "nortest") (Fernandez, 1992). Distribution normality was considered as acceptable for $P > 0.01$. Results are expressed as means ± SDs.

3.4. Results

A total of 27 infants were assessed for eligibility (**Figure 23**), from which 14 were included (*i.e.,* with randomization of the experimental protocol). We excluded 13 infants because of digestive troubles (n = 4), feeding conditions not in accordance with the inclusion criteria (n = 4), transfer to another hospital (n = 2), oral nutrition (n = 2), and death (n = 1). From the 14 included infants, 12 completed the 6 d of follow-up and were included in the analysis. **Table 10** summarizes the maternal and neonatal characteristics of the 12 study participants.

Table 10. Background characteristics of infants (6 males, 6 females) who completed 6 days of follow-up

	Mean ± SD	Range
Gestational age at birth (wk)	30.0 ± 1.1	28.1 – 31.7
Birth body weight (kg)	1.4 ± 0.3	0.9 – 1.9
Age at first day (d)	27 ± 12	14 – 53
Body weight at first day (kg)	1.8 ± 0.4	1.2 – 2.4

Human milk composition and microstructure

Table 11 displays the mean macronutrient composition and FA distribution of RHM and PHM and shows large variations in macronutrient content, mainly with respect to total proteins and lipids. PHM had a greater total protein content than RHM ($P < 0.01$), whereas the lipid content did not differ between the milks ($P > 0.05$). The RHM and PHM had similar profiles with respect to the FA content and were dominated by oleic, palmitic, and linoleic acids. Only stearic acid (18:0) and ω-6/ω-3 differed, being lower in the PHM than in the RHM ($P < 0.05$). In contrast, the PHM had a higher content of docosadienoic acid (C22:2 ω-6; $P < 0.05$).

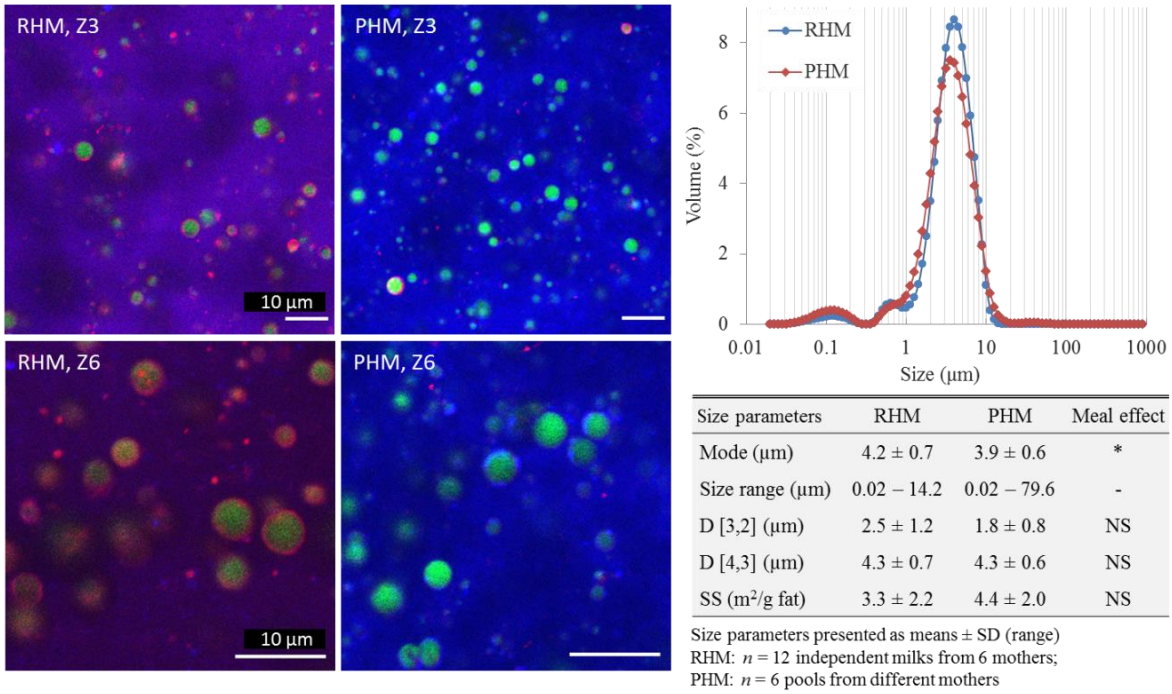
The microstructure of RHM and PHM is illustrated in **Figure 24**. The native MFG membrane surrounding the TG core was observed for both the RHM and PHM. A reorganization of proteins around the MFG membrane and in the soluble phase was observed for PHM in the CLSM images. Although the main mode diameter was smaller for PHM than RHM ($P < 0.05$), PHM contained particles in a wider size range (0.02–80 µm) than RHM (0.02–14 µm), suggesting the formation of large aggregates after pasteurization. The surface-weighted mean (D [3,2]), the volume weighted mean (D [4,3]), and specific surface area were not significantly different between the two types of milk.

Table 11. Macronutrient composition and FA distribution of RHM and PHM[1]

	Means ± SD		Meal effect[2]	Range	
	RHM	PHM		RHM	PHM
Macronutrients (g/L)					
Lipids	40.3 ± 12.9	38.6 ± 5.1	NS	12 - 80	30 - 45.5
Proteins	16 ± 3.5	20.9 ± 4.8	***	9 - 30	16 - 31
Carbohydrates	77.8 ± 5.8	68.3 ± 5.6	***	67 - 101	56 - 74
Fatty acids (% *w/w*)					
C8:0	0.3 ± 0.2	0.2 ± 0.2	NS	0 - 0.9	0 - 0.4
C10:0	1.8 ± 0.4	1.7 ± 0.4	NS	0.9 - 3.1	1.2 - 2.6
C12:0	6.5 ± 2.1	6.4 ± 1.5	NS	2.6 - 14.6	4.3 - 8.8
C14:0	7.6 ± 2.1	8 ± 2.2	NS	4.4 - 15	5.7 - 13.6
C14:1 ω-5	0.4 ± 0.1	0.4 ± 0.1	NS	0.2 - 0.7	0.2 - 0.7
C16:0	24 ± 2.8	24.4 ± 3.1	NS	16.7 - 30.9	19.3 - 29.1
C16:1 ω-5	2.7 ± 0.7	2.8 ± 0.5	NS	1 - 4.1	2 - 3.5
C18:0	6.8 ± 1.2	6.2 ± 1.3	*	4.3 - 9.9	4.4 - 8.4
C18:1 ω-9	34 ± 4	33.4 ± 3.7	NS	24 - 43.3	26.2 - 40.6
C18:2 ω-6	9.4 ± 2	8.8 ± 2.2	NS	4.5 - 14.1	6.2 - 14.5
C18:3 ω-6	0.2 ± 0.1	0.3 ± 0.2	NS	0 - 0.7	0 - 0.7
C18:3 ω-3	0.7 ± 0.5	0.8 ± 0.6	NS	0 - 2.1	0.2 - 2
C20:0	0.4 ± 0.4	0.4 ± 0.3	NS	0 - 2.9	0.1 - 1.2
C20:1 ω-9	0.4 ± 0.3	0.5 ± 0.2	NS	0 - 1.1	0.2 - 0.9
C20:2 ω-6	0.2 ± 0.2	0.3 ± 0.3	NS	0 - 1.3	0 - 0.8
C20:3 ω-6	0.3 ± 0.2	0.3 ± 0.2	NS	0 - 0.8	0 - 0.7
C20:4 ω-6	0.1 ± 0.2	0.2 ± 0.2	NS	0 - 0.7	0 - 0.6
C20:5 ω-3	0.1 ± 0.2	0.2 ± 0.3	NS	0 - 0.7	0 - 0.9
C22:0	0.1 ± 0.4	0.2 ± 0.3	NS	0 - 2.8	0 - 1
C22:1 ω-9	0.1 ± 0.2	0.2 ± 0.2	NS	0 - 0.9	0 - 0.6
C22:2 ω-6	0.1 ± 0.3	0.3 ± 0.3	*	0 - 2.3	0 - 1
C22:5 ω-3	0.2 ± 0.1	0.2 ± 0.2	NS	0 - 0.5	0 - 0.7
C22:6 ω-3	0.3 ± 0.2	0.3 ± 0.2	NS	0 - 1.2	0.1 - 1
C24:0	0.1 ± 0.1	0.2 ± 0.2	NS	0 - 0.4	0.1 - 0.8
C24:1 ω-9	0.2 ± 0.3	0.3 ± 0.3	NS	0 - 1.8	0 - 1
SFA	48.8 ± 4.8	48.8 ± 5.1	NS	39.1 - 61.1	41.1 - 57
UFA	51.2 ± 4.8	51.2 ± 5.1	NS	38.9 - 60.9	43 - 58.9
SC-FA	0.3 ± 0.2	0.2 ± 0.2	NS	0 - 0.9	0 - 0.4
MC-FA	16.4 ± 4.3	16.4 ± 3.5	NS	8.5 - 32.9	12.1 - 24.1
LC-FA	81.9 ± 4.5	81.3 ± 4.2	NS	65.8 - 90.5	71.1 - 86.4
VLC-FA	1.4 ± 1.2	2.1 ± 1.3	NS	0.4 - 8.8	0.9 - 4.6
ω-6 / ω-3	7.9 ± 4.4	6.3 ± 2.9	*	3.6 - 29.7	2.6 - 13.3

[1] Values are the ranges and means ± SD of RHM (*n* = 64 milks) and PHM (*n* = 12 pools of milk given to the 12 infants) available for analysis. FA, fatty acid; LC, long chain; MC, medium chain; PHM, pasteurized human milk; RHM, raw human milk; SC, short chain; SFA, saturated fatty acid; UFA, unsaturated fatty acid; VLC, very long chain

[2] Values compared with the use of a paired t test: $P < 0.001$ (***), $P < 0.05$ (*) and $P > 0.05$ (NS).



Size parameters	RHM	PHM	Meal effect
Mode (µm)	4.2 ± 0.7	3.9 ± 0.6	*
Size range (µm)	0.02 – 14.2	0.02 – 79.6	-
D [3,2] (µm)	2.5 ± 1.2	1.8 ± 0.8	NS
D [4,3] (µm)	4.3 ± 0.7	4.3 ± 0.6	NS
SS (m^2/g fat)	3.3 ± 2.2	4.4 ± 2.0	NS

Size parameters presented as means ± SD (range)
RHM: $n = 12$ independent milks from 6 mothers;
PHM: $n = 6$ pools from different mothers

Figure 24. Microstructure of PHM and RHM before digestion. (A) Representative CLSM images (X60 zoom 3 and 6). Scale bars, 10 µm. Proteins are colored in blue (fast green), apolar lipids are colored in green (LipidTOX), and polar lipids are colored in red (rhodamine PE). Particle size distribution (B) and associated size parameters (C) were determined with the use of laser light scattering in water. P < 0.05 (*); P > 0.05 (NS). CLSM, confocal laser scanning microscopy; PE, phosphatidylethanolamine; PHM, pasteurized human milk; RHM, raw human milk; SS, specific surface area.

Chyme characteristics: pH levels, content volume and gastric emptying rate

The volume and pH of the gastric content, which both decreased throughout the digestion of both RHM and PHM (*P* < 0.001), were not influenced by pasteurization (*P* > 0.05, **Figure 25A** and **B**). The mean values of gastric pH were 6.0 ± 0.6, 5.2 ± 0.7 and 4.4 ± 0.8 after 35, 60 and 90 min of digestion, respectively (**Figure 25A**). The mean total gastric volume was 7.9 ± 3.6, 6.7 ± 3.7 and 3.6 ± 2.7 mL/kg after 35, 60 and 90 min of digestion, respectively (**Figure 25B**).

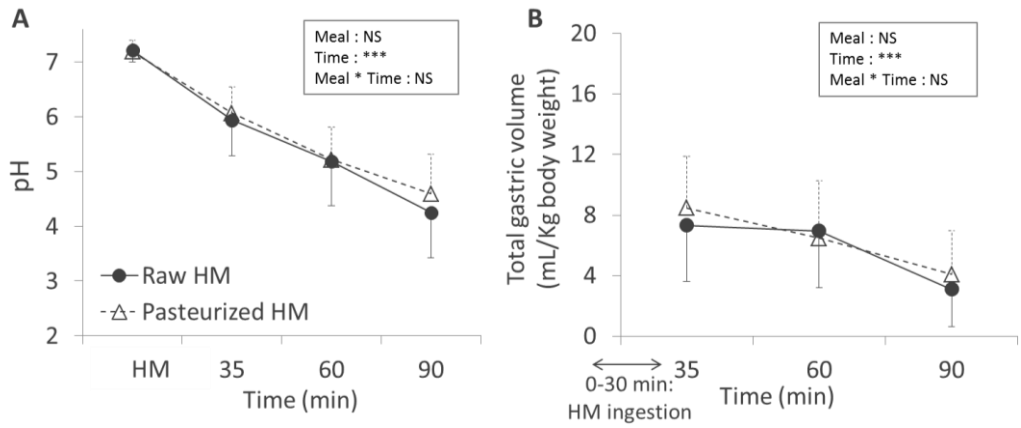


Figure 25. Changes with time in the gastric pH levels (A) and total volume (B) after ingestion of RHM (n = 58 and 67 points, respectively) and PHM (n = 65 and 69 points, respectively). Values are means ± SD. P < 0.001 (***); P < 0.01 (**); P < 0.05 (*); P > 0.05 (NS). Data from HM before digestion (A) were not included in the statistical analysis. HM, human milk.

The changes in total gastric volume over the first 35 min of digestion were estimated and are presented in **Figure 26**. The taurine marker was used to estimate the contribution of RM and gastric secretions to the total volume of gastric contents, as well as the gastric emptying rate of the meal. **Figure 26A** shows the mean estimated RM contributing to the corresponding total volume. The difference between the 2 curves corresponds to the volumes of gastric secretion present in the stomach, a rate that was quite stable during the gastric digestion: 0.95 ± 1.0, 0.91 ± 0.8 and 0.99 ± 0.8 mL/kg after 35, 60, and 90 min of digestion, respectively. However, because of gastric emptying, the proportion of secretion in the gastric volume content increased with time: 11.2 ± 10.6, 13.5 ± 9.2 and 22.8 ± 14.0% at 35, 60 and 90 min of digestion, respectively.

The rate of meal emptying during gastric digestion was not significantly different between RHM and PHM ($P > 0.05$, **Supplementary Figure 4**). The estimated mean fractions of the ingested meal remaining in the stomach were 36.1 ± 15.7, 30.9 ± 13.3 and 18.6 ± 10.9% after 35, 60, and 90 min of digestion, respectively (**Figure 26B**). Although the patterns of gastric emptying rate varied highly among infants, for 9 of 12 cases the fraction of the ingested meal remaining in the stomach was <50% at 35 min, thus indicating that the half-emptying time of the meal occurred before 35 min. The mean half-emptying time of the meal was ~30 min for the 12 infants (**Figure 26B**).

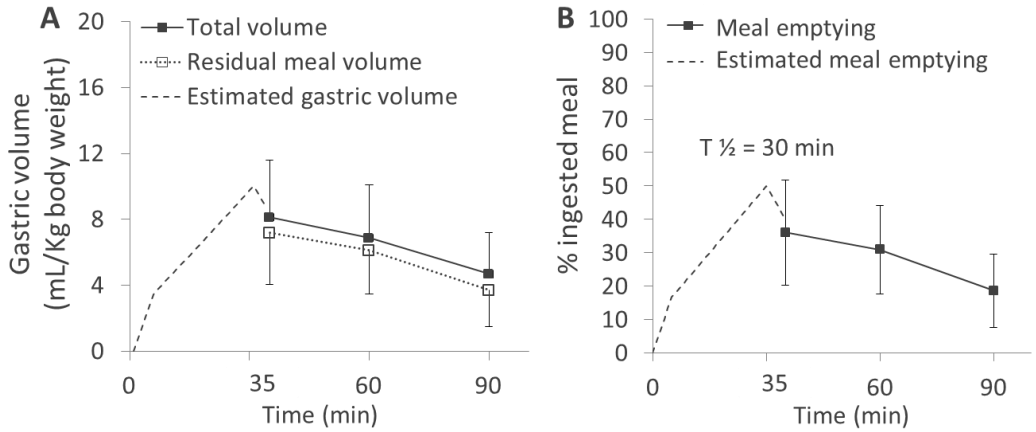


Figure 26. Contribution of residual meal to total gastric content (A) and the rate of meal emptying (B) estimated with the use of soluble-phase taurine as a meal marker. The contribution of the gastric secretions is represented by the difference between the curves for total and residual meal volumes (A). Values for the initial period from 0 to 35 min were estimated from the rate of milk ingestion (0–30 min) and emptying (5–35 min). Values are means ± SD (RHM: n = 48 points; PHM: n = 61 points). PHM, pasteurized human milk; RHM, raw human milk; T ½, half-emptying of the ingested meal.

Structural changes during gastric digestion of raw and pasteurized human milk

Figure 27 shows the CLSM images representing the mean evolution of particle size distribution of RHM and PHM during gastric digestion (n = 6 infants, mean gestational age: 29.2 ± 0.6 wk, mean body weight and age at the first day of the study: 1.6 ± 0.2 kg and 27.8 ± 14.4 days, respectively).

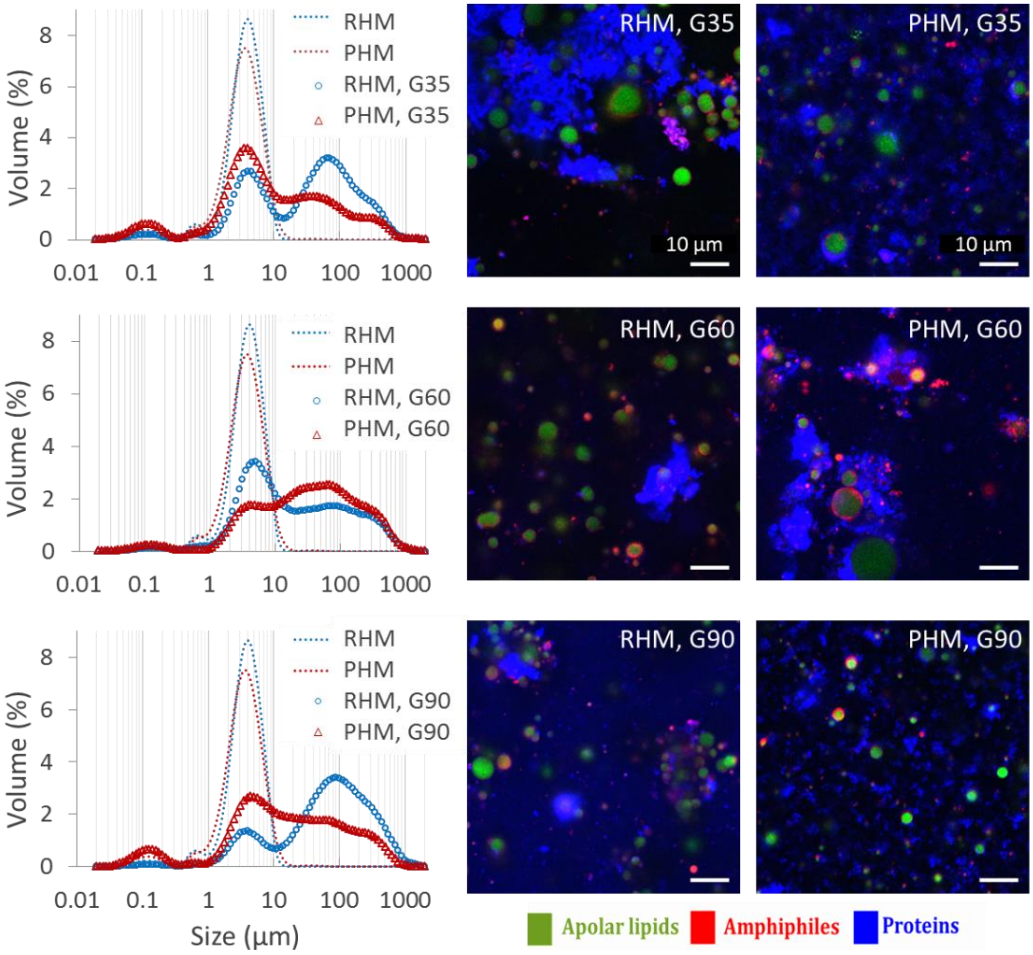


Figure 27. Changes in RHM and PHM structure during gastric digestion at 35 min (A), 60 min (B), and 90 min (C). Images are from CLSM (X60 zoom 3). Proteins are colored in blue (Fast Green), apolar lipids are colored in green (LipidTOX), and polar lipids are colored in red (rhodamine PE). Particle size distribution was determined with the use of laser light scattering in water. Data represent the mean of 6 infants; each point was measured in triplicate. CLSM, confocal laser scanning microscopy; G, gastric phase; PE, phosphatidylethanolamine; PHM, pasteurized human milk; RHM, raw human milk.

Even if the disintegration patterns of the milks varied greatly among the infants, strong emulsion destabilization and protein aggregation were systematically observed from 35 min of digestion onward for both RHM and PHM. Although the dominant particle size was ~4 µm in milk before digestion, larger aggregates were formed during digestion that resulted in bimodal or even trimodal distributions (~70–250 µm particles). At 35 min, the aggregation was less pronounced for PHM than for RHM (noting a dominant size of 4 µm compared with 90 µm). The aggregation of particles in the PHM did increase at 60 min but then fell at 90 min, which is explained by the increased contribution of particles in the 0.1–4 µm size range. These changes also affected the specific surface area, which was consequentially higher for PHM than for RHM ($P < 0.05$). At 35, 60, and 90 min, the specific surface area means were, respectively, 1.8 ± 2.4, 1.7 ± 1.3 and 0.8 ± 1.3 m^2/g for RHM and 4.7 ± 5.4, 1.8 ± 2.7 and 4.8 ± 4.9 m^2/g for PHM.

Proteolysis kinetics

Supplementary Figure 5 shows the decreasing concentrations of the major milk proteins, (*i.e.*, lactoferrin, serum albumin, α-lactalbumin and β-casein during the gastric digestion of RHM and PHM. The decrease in concentration was caused by proteolysis as well as by meal emptying and dilution by secretions. Pasteurization did not significantly affect the decrease of serum albumin and β-casein ($P > 0.05$). However, the decrease of lactoferrin was faster for PHM than RHM ($P < 0.01$). On the contrary, the decrease of α-lactalbumin was slower for PHM than RHM, but only at 90 min of digestion ($P < 0.05$). The distribution normality for α-lactalbumin residuals was considered as acceptable with P = 0.047.

After correcting the decrease in concentration of intact proteins for meal emptying and dilution by gastric secretions with the use of the RM, it was observed that proteolysis occurred for the 4 major milk proteins ($P < 0.001$) (**Figure 28**). β-Casein was proteolyzed faster, whereas α-lactalbumin was the most resistant to gastric digestion. Such correction did not change the statistical differences between RHM and PHM.

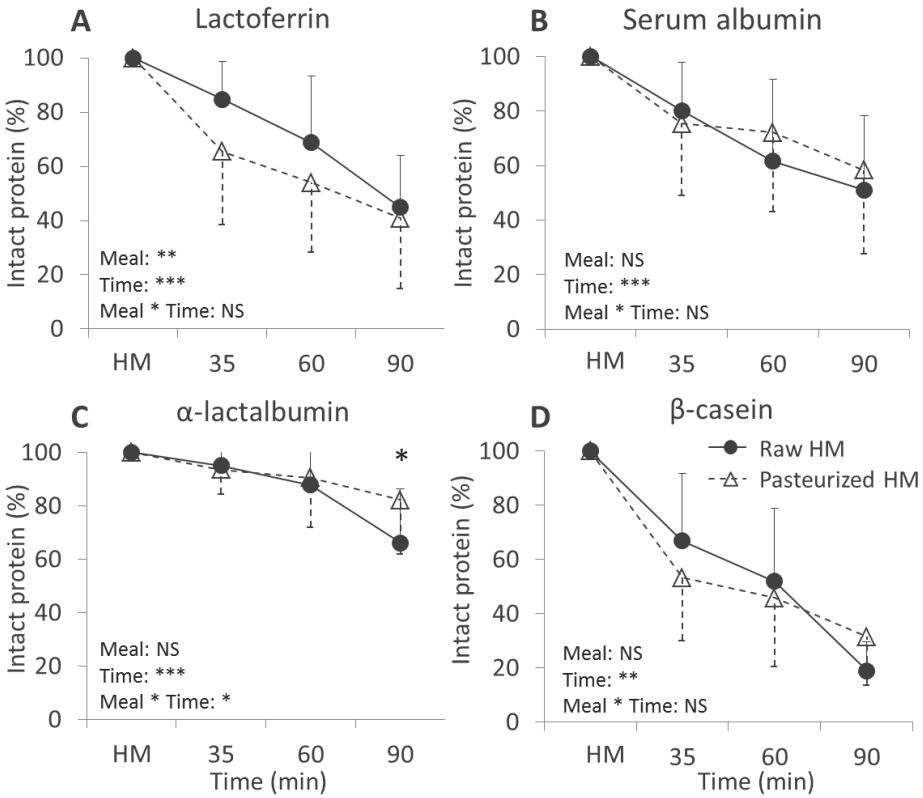


Figure 28. Kinetics of proteolysis of raw HM and pasteurized HM during gastric digestion: lactoferrin (A), serum albumin (B), α-lactalbumin (C), and β-casein (D). The percentage of each intact protein remaining in the stomach at a given time was estimated by comparison with the corresponding HM and corrected for meal dilution and emptying with the use of soluble-phase taurine as a meal marker. Values are means ± SD (raw HM: n = 54 points; pasteurized HM: n = 65 points). P < 0.001 (***); P < 0.01 (**); P < 0.05 (*); P > 0.05 (NS). Data from milk before digestion were not included in the statistical analysis. HM, human milk.

Lipolysis kinetics and fatty acid release

The milk pre-lipolysis and the kinetics of instantaneous lipolysis are presented in **Figure 29A.** The pre-lipolysis was analyzed in human milk used for 4 infants (23 independent expressed milks for RHM and 4 pools of PHM). The pre-lipolysis degree varied greatly among the RHM samples (mean: 2.2% ± 0.8%; range: 0.9–3.8%) and was lower overall ($P < 0.001$) than that found in the PHM (mean: 3.2% ± 0.6%; range: 2.4–3.9%). During digestion, the instantaneous lipolysis level increased over time ($P < 0.001$) but was not significantly affected by pasteurization ($P > 0.05$), with means of 8.7% ± 4.4%, 11.4% ± 4.7%, and 12.6% ± 4.7% at 35, 60, and 90 min, respectively. After 90 min of gastric digestion, the highest level of instantaneous lipolysis observed was 28.0%. This instantaneous lipolysis level was not statistically correlated to the gastric volume remaining in the stomach. Considering the global lipolysis from 35 to 90 min (*i.e.*, the total amount of FFAs released from milk TGs during this period), it was not changed by pasteurization ($P > 0.05$), but high interindividual variations from 35 to 90 min were noted (mean: 4.3% ± 2.7%; range: 0.9–10.9%).

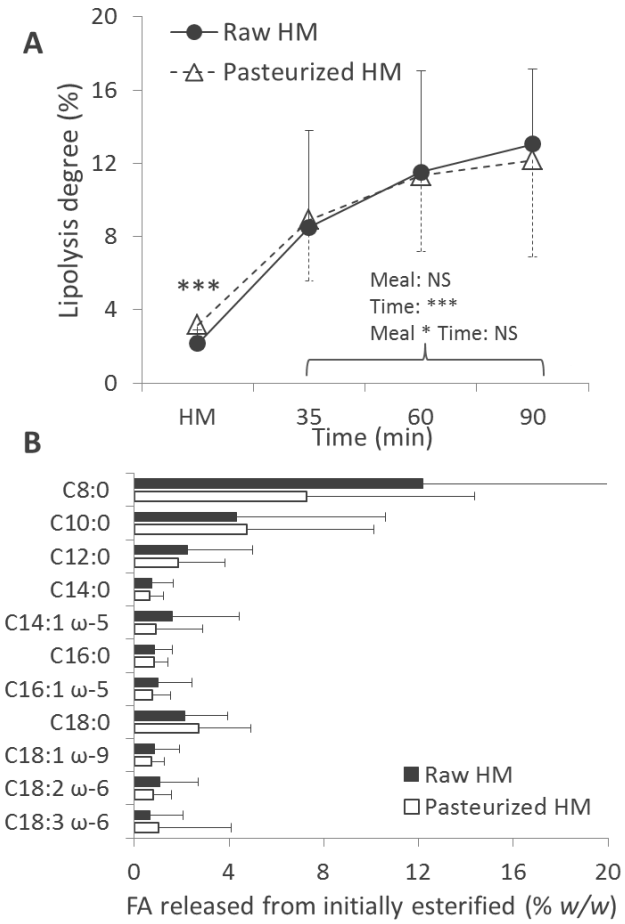


Figure 29. Kinetics of RHM and PHM lipolysis during gastric digestion (A). The instantaneous lipolysis degree (concentration of FFAs compared with total FA composition at a given time) was based on thin-layer chromatography coupled to a flame ionization detector for digesta and on gas chromatography for undigested milk. Statistical analysis of data from milk before digestion (HM: 4 infants; RHM: 23 independent milks; PHM: 4 pools of milk) was performed separately from kinetic points (n = 12 infants; 57 points for RHM and 65 points for PHM). P < 0.001 (***); P < 0.01 (**); P < 0.05 (*); P > 0.05 (NS). (B) FAs released from RHM and PHM at 90

min of gastric digestion (% *wt:wt* of each FFA present in the stomach at 90 min compared with the total FAs ingested) determined based on gas chromatography. For each FA, n = 16 points for RHM and n = 20 points for PHM. Values are means ± SD. FA, fatty acid; FFA, free fatty acid; HM, human milk; PHM, pasteurized human milk; RHM, raw human milk.

Figure 29B shows the bioaccessibility of some FAs at 90 min of gastric digestion. Globally, the highest percentage of release was for C8:0, followed by C10:0, C12:0, and C18:0. The bioaccessibility of these FAs at 90 min was not affected by pasteurization ($P > 0.05$).

3.5. Discussion

This study was the first randomized controlled trial to our knowledge to evaluate the impact of pasteurization on the kinetics of gastric digestion and the disintegration of human milk. We demonstrated that pasteurization affected the structural disintegration of the emulsion and the kinetics of hydrolysis of both lactoferrin and, to a lesser extent, α-lactalbumin. However, although previously suggested by several authors (Lindquist & Hernell, 2010; Armand et al., 1996; Hamosh et al., 1981; Hamosh, Sivasubramanian, Salzman-Mann, & Hamosh, 1978), the hypothesis that the thermal inactivation of BSSL affects the gastric lipolysis was not supported. Two particular strengths of this study are that each infant was his or her own control in assessing the effect of pasteurization on gastric digestion and that the content of lipids and proteins of each milk was taken into account in the statistical analysis.

In this study, the lipolysis level before ingestion was higher in PHM than for RHM, which is likely explained by the action of the BSSL facilitated by the MFG being damaged after freeze-thaw cycles. The fresh RHM was also exposed to the action of the BSSL, but to a lesser extent because the refrigeration time was <24 h and did not include a freeze-thaw cycle. At the beginning of the gastric digestion of RHM, some activity of the BSSL is possible because the pH measured was close to its optimal pH range (6.5–7.5) (Bakala N'Goma et al., 2012), but this is probably limited because human milk only contains small amount of bile salts (Forsyth et al., 1990). However, noting a decrease in pH and a specific activity largely favoring human gastric lipase (HGL) over BSSL (Bakala N'Goma et al., 2012; Gargouri et al., 1986), the activity of the BSSL was likely to be surpassed by that of HGL. This could explain the lack of any difference in the instantaneous lipolysis moving from 35 to 90 min of gastric digestion.

Nevertheless, the contribution of BSSL to fat digestion may be greater in intestinal digestion than observed in pre-digestion or gastric digestion, reflecting a more favorable environment (pH and bile salt concentrations) (Bourlieu et al., 2014; Lindquist & Hernell, 2010). This contribution would be important because human pancreatic lipase is immature in preterm infants and because the intestinal phase of digestion is essential for nutrient uptake and bioavailability (Bourlieu et al., 2014).

Indeed, in a previous *in vitro* study that simulated preterm newborn digestion, the intestinal lipolysis, unlike gastric lipolysis, tended to be higher (P = 0.056) for RHM than for PHM (de Oliveira et al., 2016). In addition, the broad range of substrates that BSSL can digest reinforces its biological role in the preterm: BSSL can hydrolyze *sn*-2 MGs to FAs (unlike HGL and human pancreatic lipase), which are more readily absorbed than MGs under conditions of low intraluminal concentration of bile salts that are typical of preterm newborns (Bernback et al., 1990). Recent *in vivo* data from a randomized placebo-controlled study (Casper et al., 2016) indicated no substantial improvement in infant growth velocity with recombinant human BSSL addition to infant formulas or to PHM. However, a positive effect was identified when only infant formula without medium-chain FAs was administered or when a small-for-gestational-age subgroup was considered (Casper et al., 2014); this finding is consistent with older studies on very-low-birth-weight infants, indicating that pasteurization, through the inactivation of BSSL, reduced fat absorption from human milk by ~33% (Andersson et al., 2007; Williamson et al., 1978).

No difference was found in the bioaccessibility of different FAs released from RHM and PHM at 90 min, whereas a decrease in the gastric release of short- to medium-chain FAs (C8:0 to C12:0, C14:0, C14:1; $P < 0.05$) was previously observed *in vitro* after pasteurization (de Oliveira et al., 2016). This apparent contradiction can be explained by 1) a difference in FA-type selectivity between HGL and the rabbit gastric lipase used *in vitro* and 2) the possible absorption of FFAs by the gastric mucosa (Lemarié et al., 2016; Carey, Small, & Bliss, 1983) not simulated by the *in vitro* study. Indeed, when looking at the FAs released at 90 min, the proportions of C8:0 to C12:0 observed were much higher *in vitro* (12–95%) than *in vivo* (2–12%).

With respect to the gastric conditions, secretions were constantly produced during the digestion (~1 mL/kg), and these secretions contributed greatly to the total gastric volume (11–23% at 35 and 90 min), contradicting the even larger variations reported by Roman et al. (2007) (4–12 mL/kg and 28–48% at 30 and 90 min). In this work, the half-emptying time at 30 min is in agreement with other *in vivo* studies on preterm infants after human milk ingestion (Bourlieu et al., 2014), although a large variation was reported (25–47 min).

The emulsion disintegration patterns during digestion revealed larger aggregates formed from RHM than PHM. This difference did not reduce the rate of meal emptying for PHM, which remained the same for RHM and PHM. This observation is likely to be a positive factor because delayed gastric emptying potentially increases the risk of gastrointestinal infections, which are already high in preterm newborns (Berni Canani & Terrin, 2010).

Proteolysis occurred from the beginning of gastric digestion for both RHM and PHM, which is notable because the pepsin activity and level of conversion of pepsinogen to pepsin are supposed to be

limited at high pH levels (Bourlieu et al., 2014). However, as previously suggested by others (Dallas et al., 2014; Holton et al., 2014), this proteolysis could also result from the action of milk proteases. Pasteurization selectively affecting gastric proteolysis can be explained by the fact that the susceptibility to proteolysis varies depending on the specific structure of the protein and on its organization in the milk emulsion (Zhang et al., 2014; Chatterton et al., 2004), both of which are clearly affected by pasteurization. Indeed, the apo-form of lactoferrin has been reported to be less resistant to proteolysis than its holo-form (iron-binding) (Brines & Brock, 1983; Brock, 1980), and Ueno et al. (2016) previously reported that bovine lactoferrin was not able to solubilize iron after thermal denaturation (65 °C for 10 min). Thus, the greater proteolysis observed for lactoferrin in PHM may result from being present more as the apo-form than the holo-form, which probably leads to less iron being absorbed after the ingestion of PHM than RHM (O'Connor et al., 2015). Furthermore, a greater proteolysis of lactoferrin in PHM may also affect its protective qualities because native lactoferrin has several benefits, such as supporting intestinal health because of its bactericidal activity (Lonnerdal, 2014; Wada & Lonnerdal, 2014). However, part of this bactericidal activity may still be maintained in the PHM, noting that it has been reported that lactoferrin-derived peptides can still bind iron and exert bioactivity (Lonnerdal, 2003; Brines & Brock, 1983). To our knowledge, no studies have investigated the link between PHM ingestion and lactoferrin absorption relating to iron status in infants.

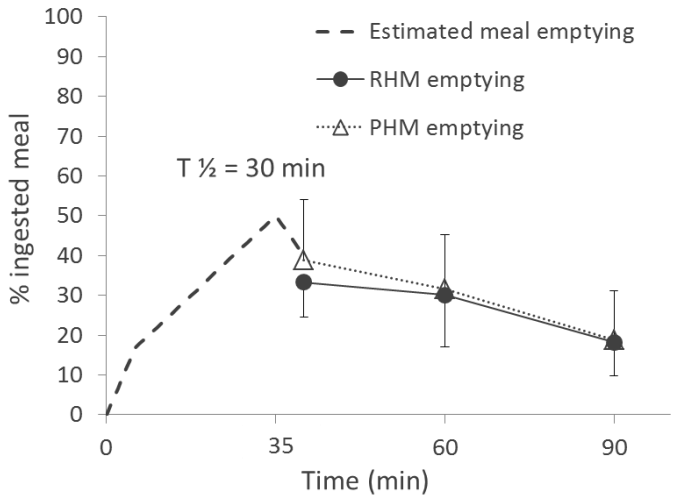
One constraint of this study was that samples were collected only after 35 min of digestion, whereas only 36% of the ingested meal was still present in the stomach at this point. However, it was not possible to take samples earlier because 30 min for ingestion had to be applied to avoid digestive intolerances. On the other hand, the estimation of the gastric volume and remaining meal during the first 35 min is new to our knowledge and underlined that Elashoff patterns do not sufficiently represent gastric emptying in preterm newborns, contrary to what has been previously suggested (Bourlieu et al., 2014; Ewer et al., 1994). This fact should be taken into account for in vitro digestion simulations. Another limitation was the slight difference in PHM and RHM composition and structure despite having collected the mothers' own milk and taken care in the design of the study and subsequent analyses to minimize this bias. These differences were certainly caused by the earlier point for milk collection for the PHM (transitional milk), which was ≥2 wk before RHM collection (mature milk).

Overall, because the gastric phase is a key step in infant digestion, it is reassuring that pasteurization did not have a major impact on gastric lipolysis and emptying, although some unobserved effects might have occurred during the first 35 min, and the possible lack of BSSL activity in the intestinal phase may have consequences in fat absorption. The already well-known impact of pasteurization on

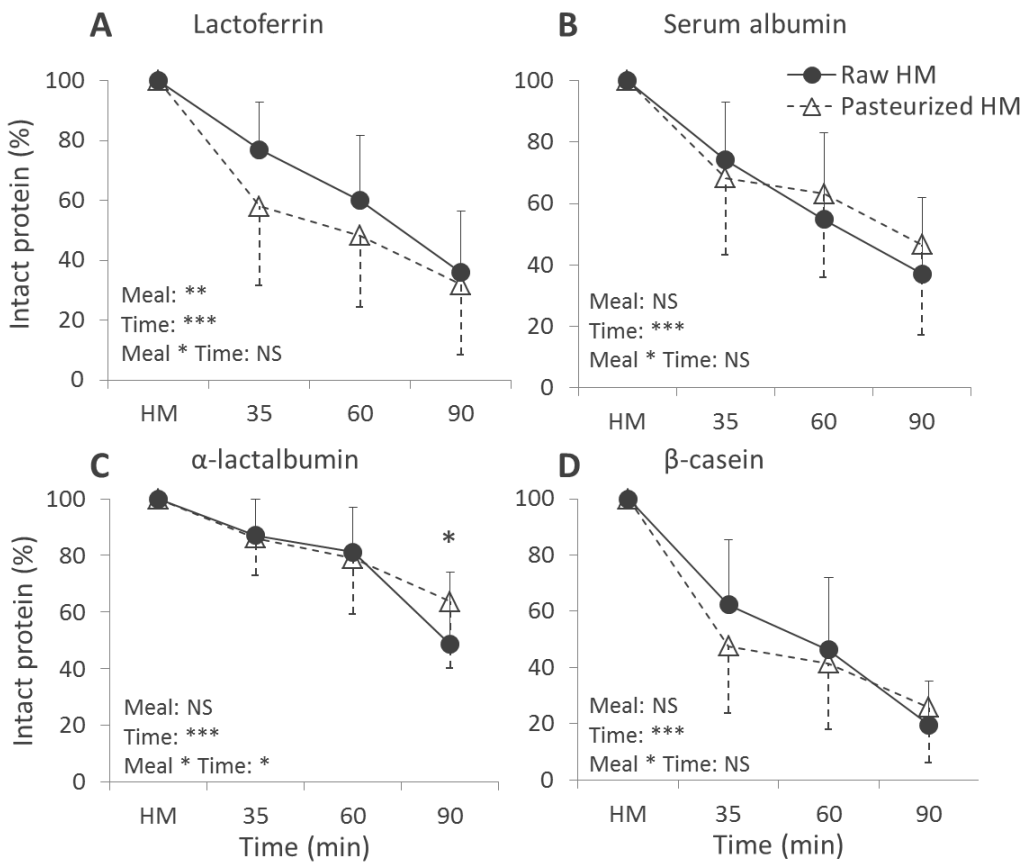
the composition of human milk (O'Connor et al., 2015), as well as our observed effects on lactoferrin and α-lactalbumin proteolysis and emulsion disintegration, should not be underestimated because they may have consequences during the subsequent intestinal digestion and absorption processes. When breastfeeding is not possible, PHM is certainly a better option for preterm infants than infant formula. However, the clinical impact of human milk pasteurization with respect to nutritional outcomes remains uncertain. The findings reported herein provide some essential knowledge for a better understanding of the preterm digestion process and for supporting the challenging decisions in clinical practices and nutritional interventions for preterm infants.

Acknowledgements: We are grateful to the participating infants and their parents. We thank Elise Thebault and Sabrina Cochennec for contributing to the experimental protocol application. We also thank Agnès Gazzola for contributing to the practical planning and implementation of the study; Clair-Yves Boquien and H´ele`ne Billard for the analyses of human milk macronutrient composition; Frédéric Carrière for contributing to the data analyses; Gwénaële Henry, Florence Rousseau, and Célia Moustiés for their laboratory work and analyses; and Mathieu Emily, Bruno Laviolle, and Alain Renault for statistical advice.

3.6. Supplementary material



Supplementary Figure 4. Rate of meal emptying for RHM and PHM, estimated using taurine (soluble phase) as a meal marker. Values between 0 and 35 min were estimated from the rate of milk ingestion (0-30 min) and emptying (5-35 min). Values are means ± SD. RHM: n = 48 points; PHM: n = 61 points. T ½, half-emptying of the ingested meal.



Supplementary Figure 5. Kinetics of decrease of major proteins during digestion of RHM and PHM. A: lactoferrin; B: serum albumin; C: α-lactalbumin; D: β-casein. The percentage of each intact protein remaining in the stomach at a given time was estimated by comparison with the corresponding human milk (HM). Values are means ± SD. For each protein, RHM: n = 54 points; PHM: n = 65 points. $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*); $P > 0.05$ (NS). Data from milk prior to digestion were not included in the statistical analysis.

Chapter 4 – Impact of homogenization of pasteurized human milk on gastric digestion in the preterm infant: a randomized controlled trial

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Randomized Controlled Trial

Impact of homogenization of pasteurized human milk on gastric digestion in the preterm infant: A randomized controlled trial

Samira C. de Oliveira [a], Amandine Bellanger [b, c], Olivia Ménard [a], Patrick Pladys [b, c], Yann Le Gouar [a], Gwénaële Henry [a], Emelyne Dirson [b], Florence Rousseau [a], Frédéric Carrière [d], Didier Dupont [a], Claire Bourlieu [a], Amélie Deglaire [a, *]

[a] STLO, Agrocampus Ouest, INRA, Rennes, France
[b] CHU Rennes, Pediatrics Department, Rennes, France
[c] University of Rennes 1, Faculty of Medicine, Rennes, France
[d] CNRS, Aix Marseille University, UMR7282 Enzymologie Interfaciale & Physiologie de la Lipolyse, Marseille, France

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As discussed in the bibliographic review (chapter 1, section 1.3), it has been suggested that ultrasound homogenization of Holder-pasteurized HM could improve fat absorption and weight gain in preterm infants, mainly by increasing the specific surface available for lipases adsorption. However, the digestive behavior of pasteurized-homogenized HM (PHHM) has never been studied. Thus, the main goal of this chapter was to answer the following question:

Does ultrasonic homogenization impact, in vivo, the gastric digestion of pasteurized HM in preterm infants?

This *in vivo* study was also part of the ARCHILACT clinical trial (Clinical Trials NCT02112331) as an independent study, and corresponded to infants included in the group B (*i.e.* receiving exclusively banked-HM from anonymous donors). For this group, eight infants were studied.

Contrary to Holder pasteurization, the ultrasonic homogenization is not practiced in the routine of the HMB. Thus, our main concern was not to add a potential hazard or risk of contamination to the HM processing. The method chosen was then an indirect ultrasonic homogenization after Holder pasteurization, *i.e.* immersing the hermetic milk bottle in a water bath right after the pasteurization, without direct contact with the milk. Then tests were performed to determine the set of parameters of sonication (*i.e.* power level, sonication amplitude, time, etc.) capable of efficiently homogenize HM, based on a condition of reducing the mode average particle size to 0.5 – 1.0 µm.

Gastric kinetics of lipolysis, proteolysis and emulsion disintegration of the different milks were assessed. Gastric volume, pH and gastric emptying rate were described.

The article originated from this study is published as a research paper at Clinical Nutrition ESPEN.

4.1. Abstract

Background & aims: It has been suggested that homogenization of Holder-pasteurized human milk (PHM) could improve fat absorption and weight gain in preterm infants, but the impact on the PHM digestive kinetics has never been studied. Our objective was to determine the impact of PHM homogenization on gastric digestion in preterm infants.

Methods: In a randomized controlled trial, eight hospitalized tube-fed preterm infants were their own control to compare the gastric digestion of PHM and of homogenized PHM (PHHM). PHM was obtained from donors and, for half of it, was homogenized by ultrasonication. Over a six-day sequence, gastric aspirates were collected twice a day, before and 35, 60 or 90 min after the start of PHM or PHHM ingestion. The impact of homogenization on PHM digestive kinetics and disintegration was tested using a general linear mixed model. Results were expressed as means ± SDs.

Results: Homogenization leaded to a six-fold increase in the specific surface ($P < 0.01$) of lipid droplets. The types of aggregates formed during digestion were different between PHM and PHHM, but the lipid fraction kept its initial structure all over the gastric digestion (native globules in PHM *vs.* blend of droplets in PHHM). Homogenization increased the gastric lipolysis level ($P < 0.01$), particularly at 35 and 60 min (22 and 24% higher for PHHM, respectively). Homogenization enhanced the proteolysis of serum albumin ($P < 0.05$) and reduced the meal emptying rate ($P < 0.001$, half-time estimated at 30 min for PHM and 38 min for PHHM). The postprandial gastric pH was not affected (4.7 ± 0.9 at 90 min).

Conclusions: Homogenization of PHM increased the gastric lipolysis level. This could be a potential strategy to improve fat absorption, and thus growth and development in infants fed with PHM; however, its gastrointestinal tolerance needs to be investigated further.

This trial was registered at clinicaltrials.gov as NCT02112331

Keywords: Digestion, Holder pasteurization, homogenization, human milk, preterm infant.

4.2. Introduction

Progress in neonatal care over the last decades, including the establishment of human milk banks (HMB), has reduced morbidity and improved the survival of vulnerable and high-risk infants, such as preterm and very low birth weight (VLBW) neonates. Ensuring adequate postnatal growth is crucial for better nutritional and clinical outcomes of preterm neonates (WHO, 2015), mainly avoiding impaired neurological development and reducing the risk of adverse metabolic consequences (Lafeber, van de Lagemaat, Rotteveel, & van, 2013; Ehrenkranz et al., 2006). However, an important part of these infants still experiences postnatal growth restrictions (Horbar et al., 2015).

Human milk given either fresh (from the own mother) or pasteurized (mainly from donors) offers several benefits and better outcomes than infant formulas (Victora et al., 2016; Arslanoglu et al., 2013). However, achieving the high requirement in proteins, energy and micronutrients for preterm newborns remains a challenge. Firstly, most of the donors of banked-HM deliver at term and are at advanced stage of lactation, which usually does not correspond to the specific requirements of the receiving babies in neonatal care services (preterm newborns and VLBW infants) (Gidrewicz & Fenton, 2014; Ballard & Morrow, 2013). Secondly, steps following expression (*e.g.* freeze-thaw cycles and Holder pasteurization) affect both the milk structure and its nutritional quality (de Oliveira et al., 2016; Peila et al., 2016; O'Connor et al., 2015). Finally, nutrient losses occur by adherence during tube feeding (Rayyan et al., 2015).

In this context, fortification of HM is considered as a strategy to assure adequate postnatal growth rate (Arslanoglu et al., 2013), although this practice remains a source of debate (Morlacchi et al., 2016; Raiten et al., 2016; Rochow et al., 2015; Adamkin & Radmacher, 2014). Another complementary strategy could be the enrichment of pasteurized human milk in recombinant bile-salt stimulated lipase (BSSL), an enzyme present in human milk that can contribute to fat digestion in neonates but that is inactivated during pasteurization. However, recent clinical trials have shown limited clinical beneficial outcomes of this practice (Casper et al., 2014; Casper et al., 2016).

Some authors have suggested the homogenization of banked-PHM as a strategy for improving fat absorption (Thomaz et al., 1999) and weight gain (Rayol et al., 1993) in preterm infants fed with PHM. Homogenization by ultrasonication fragments the native milk fat globule membrane (MFG, mean diameter of 4 µm) in smaller and uniformly distributed lipid droplets (from 0.1 to 1 µm), and leads to an increase in the surface available for digestive enzymes adsorption (Bourlieu et al., 2015b). This structural modification in the lipid droplets size could increase the digestibility of milk fat and compensate for the heat-denaturation of BSSL (Henderson et al., 1998). Additionally, it could minimize the nutrients losses during tube feeding (Rayol et al., 1993; Martinez et al., 1987). However, the impact of homogenization of PHM on its digestive kinetics has never been studied.

In this context, our objective was to investigate the impact of homogenization of PHM from donors on its *in vivo* gastric digestion in preterm infants. More specifically, we studied the gastric functions, the kinetics of lipolysis, the proteolysis and the structural disintegration of pasteurized or pasteurized and homogenized human milk.

4.3. Subjects and methods

Study design and participants

This study was a prospective randomized controlled trial (NCT02112331) conducted from April 2014 to August 2015 at the pediatric department of the University Hospital Center of Rennes, France, after approval by the Institutional Review Board of University Hospital Center of Nantes (CPP Ouest IV), France.

Supplementary Figure 7 shows the flow diagram of the infants included, as well as the criteria for eligibility and inclusion in the study. Briefly, hospitalized preterm newborns were pre-included in the study, after their parents received oral and written information about the study by the pediatricians, and gave informed written consent within 72 h from birth. Some pre-inclusion criteria were used to determine eligibility into the study: 1) gestational age at birth < 32 wk, 2) a postpartum hospital stay in the Hospital Center of Rennes, 3) nasogastric tube feeding, 4) no mother's intention or no possibility to breastfeed during the hospital stay. A medical visit of inclusion in the study was realized since the infant reached an enteral supply of at least 120 mL/kg/day with a 3 h interval feeding. Non-inclusion and exclusion criteria were: 1) digestive significant disease or malformation, including previous history of necrotizing enterocolitis, 2) abdominal bloating with abdominal tension during experimental protocol, 3) treatment with catecholamine or morphine during experimental protocol, 4) sampling failure (volume < 4 mL) on two samples taken at the same postprandial time point (except 90 min) and for the same type of milk.

Each infant was his own control to compare the gastric digestion of pasteurized human milk and pasteurized-homogenized human milk (PHM and PHHM, respectively).

Test meals

Anonymous donors collected their milk following the recommendations of the HMB. Milk was stored at −20 °C after collection and then transferred to the HMB. Milk from different collections from the same mother were thawed in a 4 °C temperature controlled room over less than 24 h, pooled and divided into bottles of 60 to 75 mL. All the bottles underwent Holder pasteurization (62.5 °C, 30 min). Half of the pool, called pasteurized human milk (PHM), went back to storage at −20 °C. The other half underwent indirect discontinuous long ultrasonic homogenization using a Q700 Sonicator (QSonica,

Newtown, USA) at a power of 595 W (three periods of 5 min interrupted by 30s of pause). This homogenized milk, called PHHM, was stored at −20 °C following the usual HBM procedures. During the period of the study, four pools of human milk were treated and each infant received test meals from a single pool.

Bacteriological tests were performed on two samples from each pool: one taken after pooling the milk and another one taken right after pasteurization. Milk was considered as adequate for consumption if the total aerobic bacterial load at 37 °C was <1 X 10^6 CFU/mL and coagulase-positive Staphylococci were <1 X 10^4 CFU/mL for the pooled milk before pasteurization and total aerobic bacterial load at 37 °C was <2 CFU/mL for the milk after pasteurization.

Before each administration, a bottle was thawed in a 4 °C temperature controlled room over a period not exceeding 24 h.

Experimental protocol, Handling of samples, Analytic methods, Calculations

All these procedures were detailed in the previous chapter, section 3.3. The only difference regards the types of milk used: in the present chapter, PHM and PHHM were compared.

Statistical analyses

Statistical analyses were conducted using the R software, version 3.1.2 (R Core Team, 2014). A paired *t* test was conducted to assess the impact of homogenization on human milk (prior to administration) and on the FA release from PHM and PHHM at 90 min of digestion. The variables concerning undigested human milk prior to use were size parameters, degree of pre-lipolysis (lipolysis of milk prior to digestion triggered by its endogenous enzymes), macronutrient content and FA proportion.

The impact of homogenization on the digestive kinetics was tested on the response variables (Y) using a linear mixed model after a model selection starting from the initial model:

$$Y = \text{Meal} + \text{Time} + \text{Meal} \times \text{Time} + \text{Subject} + \textit{Sampling day} + \textit{Body weight} + \textit{Lipid content} + \textit{Protein content} \quad (8)$$

Subject was considered as random effect and other effects were fixed. *Body weight* corresponded to the subject body weight at each sampling day (day 1 to 6) in kg, *Lipid* and *Protein contents* referred to the composition of the ingested milk and were excluded of the models for response variables concerning either proteolysis or lipolysis, respectively. The model selection was conducted on the initial model among the covariates *Body weight*, *Sampling day*, *Lipid content* and/or *Protein content* in order to choose the most parsimonious model. This was based on the AIC criterion (the smaller is

better) using the function "stepAIC" from the package "MASS". From the selected model, an analysis of deviance (function "anova.lme" from the package "nlme") was performed using the Wald chi-square test to determine the p-values for *Meal*, *Time* and *Meal x Time*. When differences were significant (p-value < 0.05), post hoc tests were performed using a Bonferroni correction (function "glht" from the package "multcomp"). The distribution normality of the residuals from the selected linear mixed models was tested for each response variable using the Kolmogorov-Smirnov test, function "lillie.test" from the package "nortest" (Fernandez, 1992). Distribution normality was considered as not strongly violated for $P > 0.01$.

Results are expressed as means ± SDs.

4.4. Results

A total of 20 infants were assessed for eligibility, from which 10 were included (*i.e.* with randomization of the experimental protocol). The exclusion of 10 infants was due to: feeding conditions not in accordance with the inclusion criteria ($n = 6$); digestive troubles ($n = 2$); and transfer to another hospital ($n = 2$). From the 10 included infants, 8 completed the six days of follow-up and were included in the present analysis. Exclusions after randomization ($n = 2$) were decided due to repeated (twice) failure in digesta collection at a given postprandial time (35 or 60) for the same type of milk. **Table 12** summarizes the maternal and neonatal characteristics of the 8 participants.

Table 12. Background characteristics of infants who completed the 6 days of follow-up ($n = 8$)

	Mean ± SD	Range
Gestational age (wk)	29.5 ± 1.5	27.3 – 31.3
Birth body weight (kg)	1.2 ± 0.3	0.8 – 1.9
Sex, *n* F/M (%)	-	5/3 (62.5)
Age at 1st day (d)	32.1 ± 21.3	11.0 – 73.0
Body weight at 1st day (kg)	1.7 ± 0.5	1.3 – 2.6

Human milk composition and microstructure

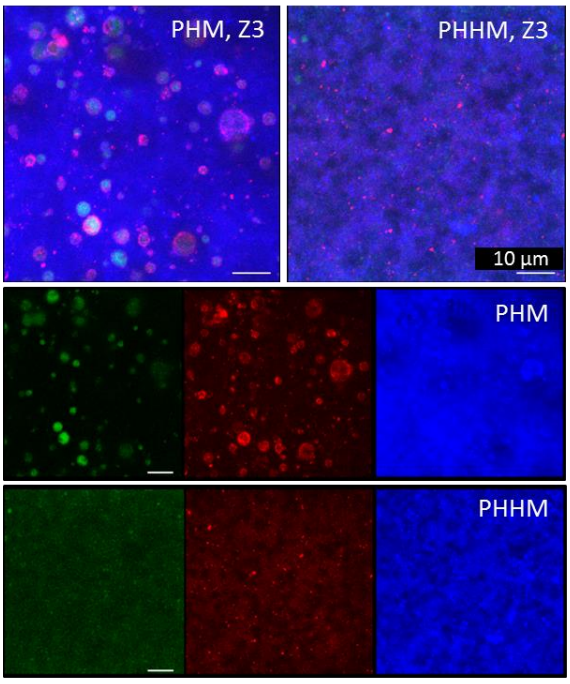
The average macronutrient composition and FA distribution in the four pools of donor milk used during the study (PHM and PHHM) are displayed in **Table 13**. No significant variations in macronutrient composition and FA contents were observed between the two types of milk ($P > 0.05$), as expected since PHHM was prepared from PHM. Lipid and protein contents averaged 31.9 ± 4.3 g/L and 10.7 ± 0.6 g/L, respectively. The FA profile was typical for human milk, dominated by oleic and palmitic acid with 29.4 ± 5.5% and 24.5 ± 2.1% (*w/w*), respectively. The average ratio ω-6/ω-3 was 6.8 ± 4.6 and saturated FA amounted to 48.7% of the total FA.

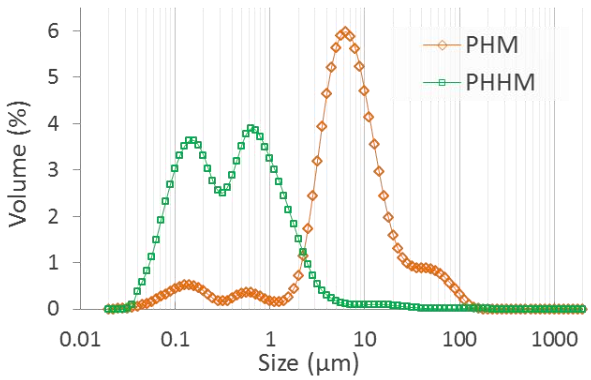
Table 13. Macronutrients composition and fatty acids distribution of pasteurized and homogenized-pasteurized human milk[1]

	PHM	PHHM
Macronutrients (g/L)		
Lipids	32.8 ± 5.0	31.0 ± 3.9
Proteins	10.7 ± 0.6	10.7 ± 0.7
Carbohydrates	78.3 ± 0.5	77.7 ± 1.2
Fatty acids (% *w/w*)		
C8:0	0.1 ± 0.2	0.1 ± 0.2
C10:0	1.8 ± 0.3	1.6 ± 0.3
C12:0	5.5 ± 1.3	5.6 ± 1.4
C14:0	7.5 ± 1.6	7.1 ± 1.6
C14:1 ω-5	0.6 ± 0.1	0.5 ± 0.3
C16:0	25.0 ± 253	24.1 ± 2.0
C16:1 ω-5	2.5 ± 1.2	2.5 ± 0.8
C18:0	8.0 ± 1.5	7.8 ± 0.7
C18:1 ω-9	28.2 ± 6.2	30.6 ± 5.4
C18:2 ω-6	9.4 ± 0.8	10.3 ± 1.2
C18:3 ω-6	0.4 ± 0.4	0.4 ± 0.3
C18:3 ω-3	1.0 ± 0.8	1.1 ± 0.6
C20:0	0.6 ± 0.4	0.5 ± 0.3
C20:1 ω-9	0.5 ± 0.4	0.5 ± 0.2
C20:2 ω-6	0.4 ± 0.5	0.4 ± 0.3
C20:3 ω-6	0.4 ± 0.4	0.4 ± 0.3
C20:4 ω-6	0.1 ± 0.1	0.0 ± 0.1
C20:5 ω-3	0.4 ± 0.4	0.3 ± 0.4
C22:0	0.1 ± 0.2	0.1 ± 0.1
C22:1 ω-9	0.5 ± 0.4	0.4 ± 0.3
C22:2 ω-6	0.4 ± 0.4	0.3 ± 0.4
C22:5 ω-3	0.4 ± 0.5	0.4 ± 0.5
C22:6 ω-3	0.5 ± 0.4	0.6 ± 0.5
C24:0	0.4 ± 0.5	0.3 ± 0.4
C24:1 ω-9	0.5 ± 0.4	0.4 ± 0.3
SFA	50.6 ± 5.0	48.3 ± 4.4
UFA	49.4 ± 5.0	51.7 ± 4.4
SC-FA	0.1 ± 0.2	0.1 ± 0.2
MC-FA	15.5 ± 3.0	14.8 ± 3.1
LC-FA	81.5 ± 3.7	82.5 ± 4.2
VLC-FA	2.9 ± 2.3	2.6 ± 2.3
ω-6 / ω-3	6.8 ± 4.7	6.8 ± 5.3

[1] Means ± SD of the PHM and PHHM given to the 8 infants (*n* = 4 pools for each meal).

The microstructure of PHM and PHHM is presented in **Figure 30**. For PHM, the native MFG with the triacylglycerol core covered by the phospholipid membrane were well observed by CLSM; heat-induced protein aggregation was observed around the MFG membrane and in the soluble phase. For PHHM, only a blend of small droplets could be observed, with no more native MFG. Regarding the distribution of the particle size, PHM presented a main mode diameter 7.0 ± 0.9 µm, and three minor peaks around 0.16, 0.6 and 50 µm. PHHM presented a bimodal distribution with peaks at 0.16 ± 0.0 and 0.8 ± 0.3 µm. The main mode diameter ($P < 0.01$), the volume weighted mean (D [4,3]) ($P < 0.01$) and the surface weighted mean (D [3,2]) ($P < 0.05$) were significantly higher for PHM compared to PHHM. As a result, a six-fold increase in the specific surface developed by the droplets was observed after homogenization ($P < 0.01$).





Size parameters	PHM	PHHM	Meal effect
Mode (µm)	7.0 ± 0.9 (5.9 - 8.2)	0.8 ± 0.3 / 0.16 ± 0.0 [a]	**
D [3,2] (µm)	1.7 ± 0.7 (1.2 - 2.7)	0.2 ± 0.0 (0.2 - 0.3)	*
D [4,3] (µm)	12.6 ± 3.5 (9.9 - 17.7)	1.0 ± 0.6 (0.5 - 1.9)	**
SS (m^2/g fat)	4.1 ± 1.2 (2.4 - 5.1)	25.5 ± 3.8 (21.5 - 28.9)	**

Size parameters presented as means ± SD (range)
n = 4 independent pools of human milk
[a] Two main mode averages separated by bars

Figure 30. Microstructure of undigested PHM and PHHM. Representative CLSM images (x60 zoom 3) with three probes and for each probe. Scale bars = 10 µm. Proteins are colored in blue (FastGreen®), apolar lipids in green (Lipidtox®) and polar lipids in red (Rhodamine-PE®). Particle size distribution and associated size parameters were determined by laser light scattering in water. SS, specific surface area. P < 0.001 (***); P < 0.01 (**); P < 0.05 (*); P > 0.05 (NS).

Chyme characteristics: pH levels, gastric content volume and emptying rate

Figure 31 presents the gastric pH and total volume during the digestion of PHM and PHHM. The gastric pH decreased through the digestion ($P < 0.001$) but was not impacted by homogenization ($P > 0.05$), averaging 5.9 ± 0.5, 5.1 ± 0.7 and 4.7 ± 0.9 after 35, 60 and 90 min of digestion, respectively (**Figure 31A**). In contrast, the postprandial gastric volume (**Figure 31B**) was significantly higher for PHHM than for PHM after 35 min of digestion (respectively 11.0 ± 3.6 and 6.8 ± 3.0 mL/kg, $P < 0.01$).

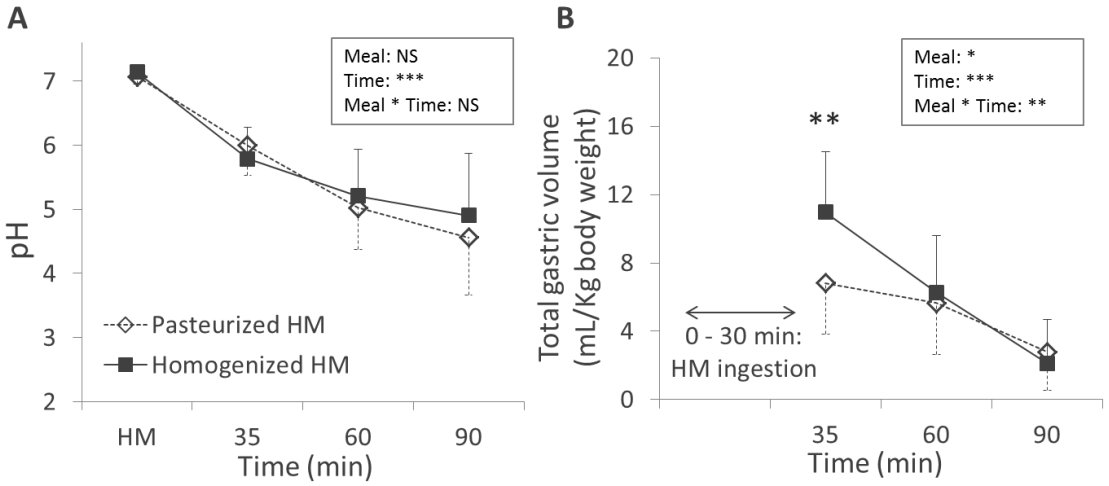


Figure 31. Changes with time in the gastric pH levels (A) and total gastric volume (B) after ingestion of PHM and PHHM. $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*); $P > 0.05$ (NS). Data from undigested human milk (HM) were not included in the statistical analysis.

The changes in the total gastric volume during the first 35 min of digestion were estimated, and the taurine marker was used in order to estimate the contribution of residual meal and gastric secretions to the total volume of gastric contents, as well as the emptying rate of the meal (**Figure 32**). The average contribution of residual meal to the corresponding total volume throughout the digestion is presented in **Figure 32A**. The difference between the two curves represents the volume of gastric secretion in the stomach, the contribution of which to the gastric volume increased with time: 8.3 ± 9.6, 13.5 ± 11.9 and 20.2 ± 17.4% of the total gastric volume after 35, 60 and 90 min of digestion, respectively.

The homogenization slowed down the meal emptying rate during the gastric digestion ($P < 0.001$, **Figure 32B**). For the two types of milk, the ingested meal remaining in the stomach was estimated to reach a maximum level after 30 min (48.2% and 65.3% of ingested PHM and PHHM, respectively). On average, the half-emptying of the meal was estimated at 30 min for PHM and 38 min for PHHM. After 90 min of digestion, on average 16.3 ± 9.4% and 21.1 ± 9.2% of the ingested PHM and PHHM, respectively, remained in the stomach.

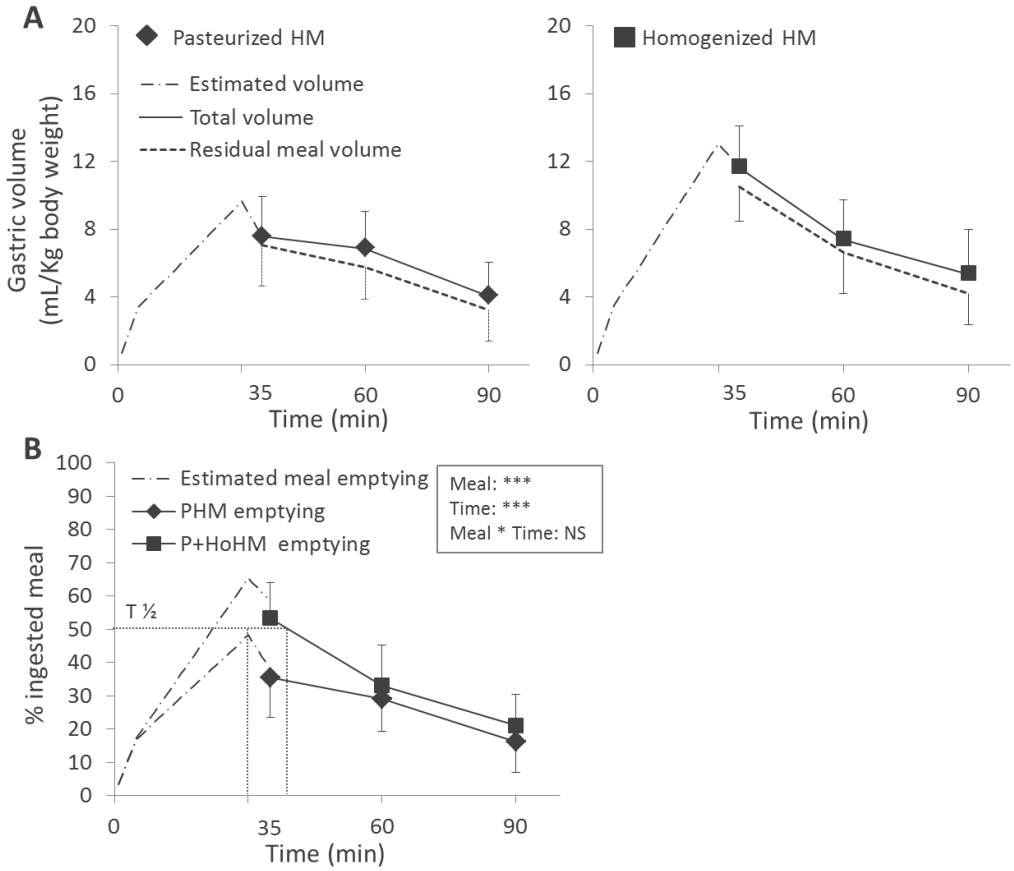


Figure 32. A) Contribution of residual meal to the total gastric contents after ingestion of PHM and PHHM. The contribution of the gastric secretions is represented by the difference between the curves of total and residual meal volumes. B) Rate of meal emptying for PHM and PHHM. P < 0.001 (***); P < 0.01 (**); P < 0.05 (*); P > 0.05 (NS). Data from values between 0 and 35 min were not included in the statistical analysis. Values between 0 and 35 min were estimated from the rate of milk ingestion (0-30 min) and emptying (5-35 min), using taurine (soluble phase) as a meal marker. T ½, half-emptying of the ingested meal. Due to quantity restrictions for taurine quantification (meal marker present in the soluble phase), the estimations of residual meal, secretions and gastric emptying rate were possible on 68 out of the 96 postprandial samples.

Structural changes during gastric digestion

Figure 33 shows the CLSM images representing the average evolution of particle size distribution in PHM and PHHM during gastric digestion (*n* = 5 infants, gestational age on average 29.7 ± 1.1 wk, body weight and age at the first day of the study on average 1.5 ± 0.2 kg and 27.2 ± 14.6 days, respectively).

The digestion patterns were marked by strong emulsion destabilization and lipid droplet aggregation, as well as protein aggregation systematically observed from 35 min onwards, for both PHM and PHHM. Aggregation and changes in particle size were particularly marked for the finest emulsion present in PHHM. But whatever the initial particle size, the surface weighted mean (D[3,2]), the volume weighted mean (D [4,3]) and the specific surface resulting from gastric digestion were not significantly different between the two types of milk (*P* > 0.05). For both of them, large aggregates were already formed after 35 min, and D [4,3] and D[3,2] progressively increased with time (*P* <

0.05). D [3,2] averaged 11.3 ± 9.7, 19.4 ± 11.3 and 28.6 ± 20.2 µm, and D [4,3] averaged 43.8 ± 32.5, 120.7 ± 92.5 and 147.0 ± 101.5 µm, respectively after 35, 60 and 90 min.

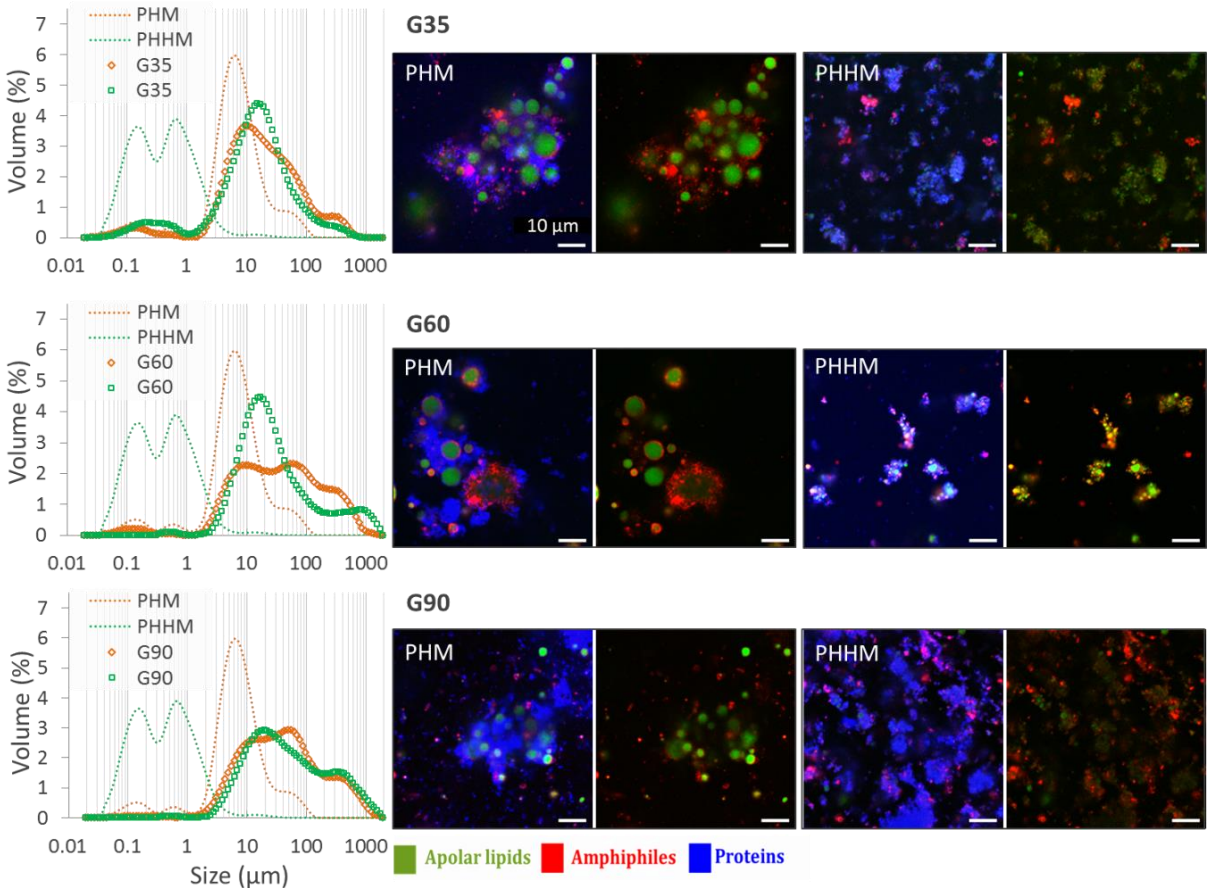


Figure 33. Disintegration of PHM and PHHM during gastric digestion. Images from CLSM (×60 zoom 3): proteins colored in blue (FastGreen®), apolar lipids in green (Lipidtox®) and polar lipids in red (Rhodamine-PE®). Each image is shown firstly with the three probes and secondly with the probes labelling only polar and apolar lipids. Particle size distribution was determined by laser light scattering in water. The latter data represents the average of five subjects (on average, gestational age of 29.7 ± 1.1 wk, body weight and age at the first day of the study of 1.5 ± 0.2 kg and 27.2 ± 14.6 days, respectively). G, gastric phase followed by time in min.

Beyond the particle size distribution, a difference in the disintegration patterns of the two meals was observed in terms of type of aggregates and of internal structure of these aggregates, using CLSM. Notably, both PHM and PHHM presented a persistence of structural characteristics of initial matrices during the whole gastric digestion. For digested PHM, the aggregates were formed by proteins and by MFG as native or partially digested lipid droplets. For digested PHHM, mixed aggregates were observed on which proteins, lipid droplets and amphiphilic molecules were colocalized (**Figure 33**).

Lipolysis kinetics and fatty acid release

Figure 34A shows the milk pre-lipolysis and the kinetics of instantaneous lipolysis during digestion**.** The pre-lipolysis was analyzed for the four pools and was similar for PHM and PHHM ($P > 0.05$), with an average value of 4.4 ± 1.0%. During digestion, the lipolysis level increased over time for both types of milk ($P < 0.001$) and was higher for PHHM than for PHM at any time of the gastric digestion ($P <$

0.01), the difference being greater at 35 and 60 min. On average, the instantaneous lipolysis levels were 8.5 ± 2.7, 9.6 ± 2.6 and 13.8 ± 5.2% for PHM *versus* 10.9 ± 1.8, 12.7 ± 2.3 and 15.0 ± 7.1% for PHHM after 35, 60 and 90 min, respectively. Likewise, the global lipolysis level from 35 to 90 min was higher for PHHM than for PHM (respectively 6.8 ± 2.0 and 3.2 ± 1.2%, $P < 0.01$).

Qualitative analysis of lipolysis products displayed in **Figure 34B**, which compares the proportion of each free FA present in the stomach at 90 min with the proportion of each FA initially esterified in human milk (% *w/w*), showed no impact of homogenization ($P > 0.05$). In both types of milk, a selective release was observed mainly for C8:0, C10:0, C12:0 and C18:0, as their relative amount were higher than that in the total esterified FA profile. Nevertheless, C16:0 and C18:1 were the FA the most predominant in the FA profile at 90 min of digestion.

Figure 34C shows the bioaccessibility of individual FA at 90 min of gastric digestion (% *w/w* of each free FA present in the stomach at 90 min *versus* the ingested amount of this FA). Globally, C10:0, C12:0, C14:1 and C18:0 presented the highest percentage of release. The bioaccessibility of C8:0 was not presented due to its limited quantity in some undigested milk (signal under the limit of quantification).

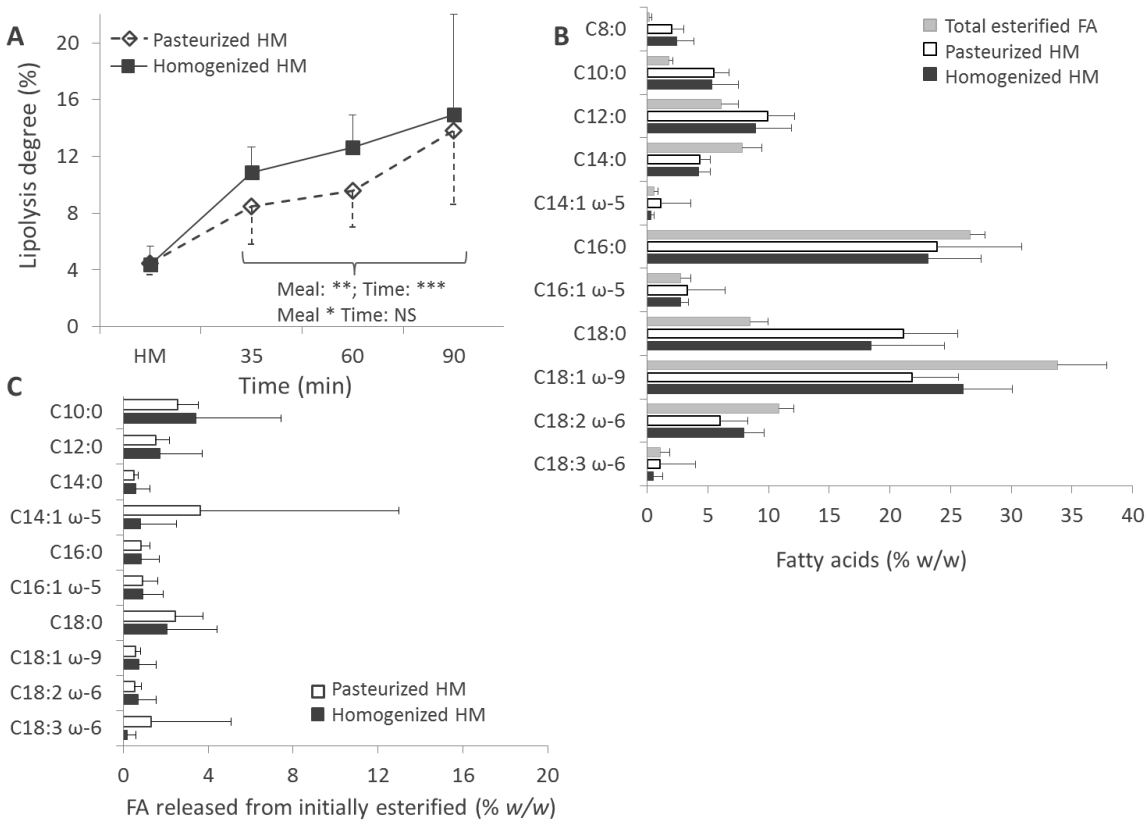


Figure 34. A) Kinetics of lipolysis of PHM and PHHM during gastric digestion. The instantaneous lipolysis degree (% level of free FA *versus* total FA composition at a given time) was determined by thin layer chromatography coupled to a flame ionization detector for digesta and on gas chromatography for undigested milk. Statistical analysis of data from undigested milk (HM, n = 4 pools) was performed separately from kinetics points. P < 0.001 (***); P < 0.01 (**); P < 0.05 (*); P > 0.05 (NS). B) Acyl chains profiles initially esterified in human milk

compared to free fatty acids released from PHM and PHHM at 90 min of gastric digestion (% *w/w* of each free FA), determined by gas chromatography. P > 0.05. C) FA released from PHM and PHHM at 90 min of gastric digestion (% *w/w* of each free FA present in the stomach at 90 min *versus* the total FA ingested), determined by gas chromatography. P > 0.05.

Proteolysis kinetics

Figure 35 shows the disappearance of the major milk proteins, *i.e.* lactoferrin, serum albumin, α-lactalbumin and β-casein during the gastric digestion of preterm PHM and PHHM. Their disappearance was due to proteolysis as well as to meal emptying and dilution by secretions. The percentage of these proteins decreased during digestion ($P < 0.001$) but was not impacted by homogenization ($P > 0.05$). In both milk, this decrease was selective: faster for β-casein and slower for α-lactalbumin, while lactoferrin and serum albumin presented intermediary patterns.

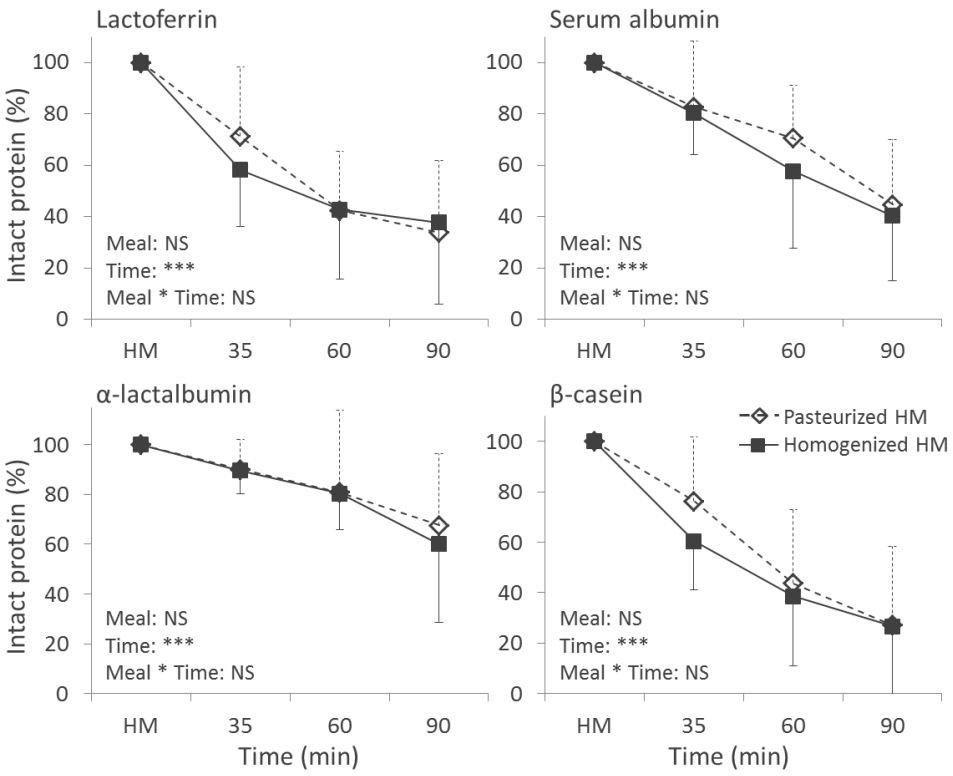


Figure 35. Kinetics of disappearance of major proteins in gastric aspirates after administration of PHM and PHHM. The percentage of each intact protein remaining in the stomach at a given time was estimated by comparison with the corresponding undigested human milk (HM). Determination was done by SDS-PAGE followed by densitometry. P < 0.001 (***); P < 0.01 (**); P < 0.05 (*); P > 0.05 (NS). Data from undigested milk were not included in the statistical analysis.

After correcting the percentage of intact proteins by meal emptying and by dilution with secretions using the residual meal estimation (**Supplementary Figure 7**), we observed a significant decrease over time of the percentage of all the intact proteins, indicating that proteolysis occurred. An exception was for α-lactalbumin ($P > 0.05$), for which the decrease did not reach statistical significance, and for which the residual distribution became not normal after correction by the

residual meal ($P < 0.001$). Only serum albumin appeared more susceptible to proteolysis in PHHM compared to PHM ($P < 0.05$).

4.4. Discussion

The present randomized controlled trial demonstrated that homogenization of PHM affected its structure before and during gastric digestion, increased the gastric lipolysis level, while increasing the gastric volume and slowing down the rate of meal emptying. To our knowledge, it is the first *in vivo* trial evaluating the impact of homogenization on the kinetics of digestion and on the disintegration of human milk in infants. Two particular strengths of this study are that the same pool of milk was used as pasteurized (PHM) or pasteurized and homogenized (PHHM), and that each infant was his or her own control in the assessment of the effect of homogenization on gastric function and digestion.

Our study demonstrated that the homogenization of pasteurized milk increased the degree of lipolysis by increasing the surface area available for lipase adsorption. Indeed, an impact of homogenization on gastric lipolysis was previously shown during *in vitro* digestion of bovine milk emulsions under conditions mimicking infant physiology (Bourlieu et al., 2015b). Furthermore, increasing the surface area is likely to delay the well-known inhibition of gastric lipase by lipolysis products, as more lipid surface is available for free FA adsorption before triggering inhibition (Garcia et al., 2014; Pafumi et al., 2002; Gargouri et al., 1986). The greater gastric lipolysis level observed for PHHM in the present study likely explains the improved fat absorption and the higher weight gain previously observed in vulnerable infants fed with homogenized compared to pasteurized human milk (Thomaz et al., 1999; Rayol et al., 1993). Another explanation could be the reduced nutrient loss during tube feeding after homogenization (Martinez et al., 1987), although this parameter was not directly evaluated in our study. In any case, homogenization seems an effective way to achieve a greater gastric lipolysis degree, and is potentially more effective than BSSL supplementation which seems to have limited clinical beneficial outcomes (Casper et al., 2016; Casper et al., 2014). However, whether a higher lipolysis in the gastric phase can favor the lipolysis and absorption in the intestinal phase remains unclear; indeed, the immature production of HPL and the low concentration of bile salts in the preterm infant could limit the absorption of lipolysis products (Bourlieu et al., 2014).

Interestingly, homogenization did not affect the individual FA release as observed at 90 min of the gastric digestion. On an absolute basis, C16:0 and C18:1 were the main FA released from milk triglycerides in the stomach for both PHM and PHHM. Nevertheless, on a relative basis, medium chain FA (C8 to C12) were preferentially released in the stomach, without taking into account that these medium chains FA might have been underestimated due to gastric absorption (de Oliveira et al., 2017a; Lemarié et al., 2016; Carey et al., 1983).

Concerning the gastric functions, the fact that homogenization did not affect the gastric acidification is in agreement with other studies reporting that, in the preterm infant, the gastric pH was not impacted by the type of meal nor by its hydrolysis degree (de Oliveira et al., 2017a; Armand et al., 1996). The higher total volume and slowing down of meal emptying rate during PHHM digestion is coherent with the higher lipolysis level observed. Indeed, the release of free FA into the duodenum is known to stimulate the secretion of cholecystokinin, an hormone that, in particular, slows down the pylorus contractions and thus the gastric emptying (Grider, 1994; Yamagishi & Debas, 1978). In the present study the internal structure of the aggregates formed was clearly different, preserving the structure type of the PHM and PHHM prior to digestion (native MFG *versus* submicronic droplets). Thus, the difference of colloidal behavior between the native MFG and submicronic droplets may also have enhanced such difference in emptying. In adults, it was demonstrated that non-stable emulsions tending to separate into an upper fatty phase and a lower liquid aqueous phase in the stomach would get emptied initially faster than a homogeneous and gastric stable emulsion (Golding & Wooster, 2010). Furthermore, the formed aggregates having different physical properties may play a role on the enzyme diffusion, which should be further investigated.

In terms of physiological impacts, the reduced gastric emptying rate of PHHM could potentially lead to an increased risk for feeding intolerance and gastrointestinal infections (Berni Canani & Terrin, 2010; Bertino et al., 2009). However, this conclusion should be treated cautiously since the half-emptying time observed for PHHM (38 min) remains close to that observed in preterm neonates fed with raw human milk (up to 47 min) and much lower than that in preterm neonates fed with infant formula (up to 72 min) (Bourlieu et al., 2014). Indeed, in these studies the higher macronutrient content in infant formulas compared to human milk likely contributed to the longer gastric emptying times, beyond the different structures.

Proteolysis was rather unaffected by homogenization, despite the expected effect of protein reorganization and adsorption onto the newly formed lipid droplets (Bermudez-Aguirre et al., 2008; Michalski & Januel, 2006), and on the contrary to previous results obtained in model infant formulas, where homogenization led to increased proteolysis (Bourlieu et al., 2015b). However, high-pressure homogenization process was applied in that study; in the present study, the ultrasound homogenization applied is more likely to increase the protein solubility by exposing their hydrophilic moieties (amino and carboxyl groups) to water, as demonstrated for bovine milk (Ashokkumar et al., 2010). Besides, Macierzanka et al. (2009) demonstrated *in vitro* that proteins in solution (β-caseins and β-lactoglobulin) were hydrolyzed slower than those adsorbed onto the lipid droplets. In the present study, only serum albumin showed an enhanced proteolysis after homogenization. Besides being a source of amino acids, the role of serum albumin in human milk is unclear (Lonnerdal, 2016).

As extensively discussed in the literature, a faster proteolysis is not necessarily advantageous since human milk intact proteins and some peptides persisting in the digestive tract may play important biologic roles for infants (Chatterton et al., 2013). Whether the faster digestion of serum albumin is positive or not for the infant remains to be elucidated; bioactive peptides released during the gastric digestion of PHM and PHHM are currently been analyzed.

The present study had some constraints. First of all, a long time of ingestion (30 min) was chosen in order to avoid digestive intolerances. Thus, the first digesta collection was realized at 35 min, when around 50% of the ingested meal was already emptied into the duodenum. Secondly, we did not study the intestinal digestive kinetics due to ethical and medical constraints concerning *in vivo* studies in infants. Although gastric digestion is a key step on infant digestion, the impact of homogenization on the intestinal digestion of human milk should also be studied *in vitro*. Lastly, the current experimental system for indirect homogenization (without risk of contamination after pasteurization) is limited to a small number of feeding bottles. Thus, the implementation of this process in the HMB routine would require the development of a higher volume system.

Taken together, our results indicate that the homogenization of pasteurized human milk increases the gastric lipolysis degree and this could be a potential strategy to improve growth and development of infants fed with pasteurized human milk in nutritional care units. Nevertheless, the nutritional outcomes and the physiological implications of this process were not evaluated here. Before concluding about the real benefits of the application of the ultrasound homogenization on pasteurized human milk, more studies are needed for carefully evaluating parameters such as digestive tolerance, risk for inflammations, impact on growth and on neurodevelopment. Importantly, the present study improves the understanding of the digestive consequences of pasteurized milk treatment by homogenization, and provides crucial knowledge allowing the setup of *in vitro* systems mimicking the physiological gastric conditions of preterm infants. Finally, it is important to keep in mind that the positive impact of homogenization refers to the Holder-pasteurized human milk digestion, which affects, on its turn, some important bioactive compounds of human milk.

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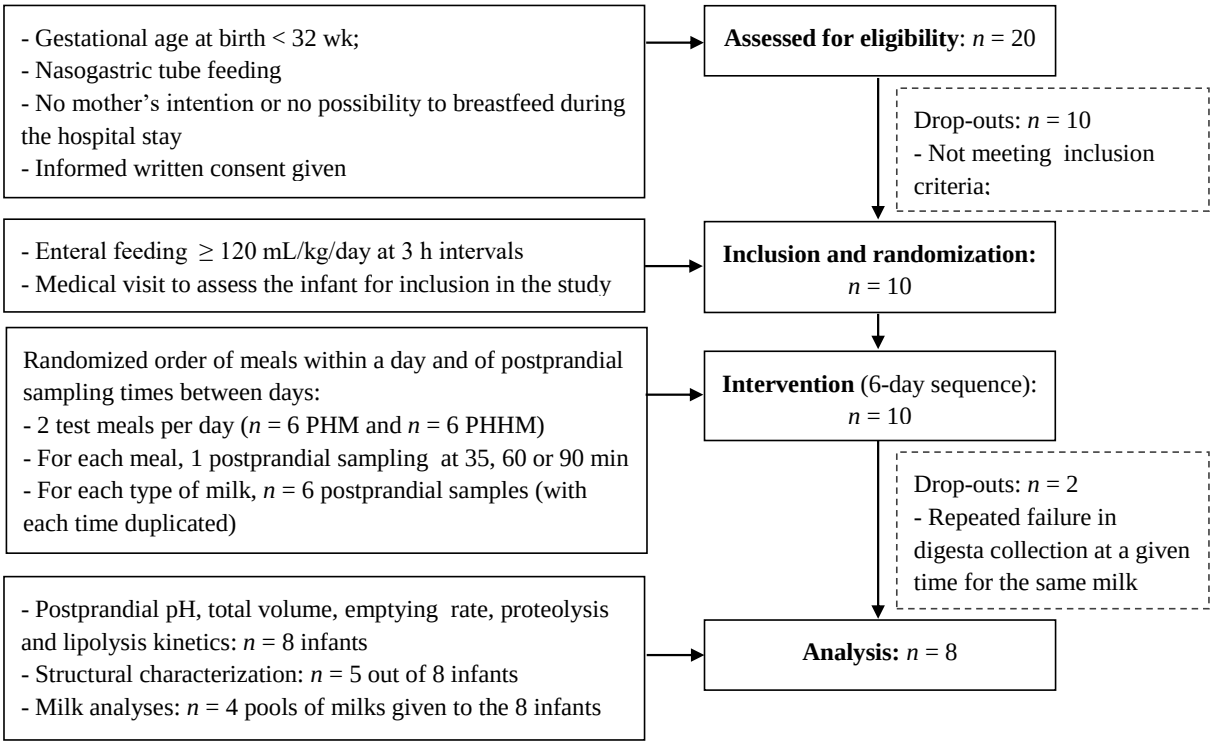
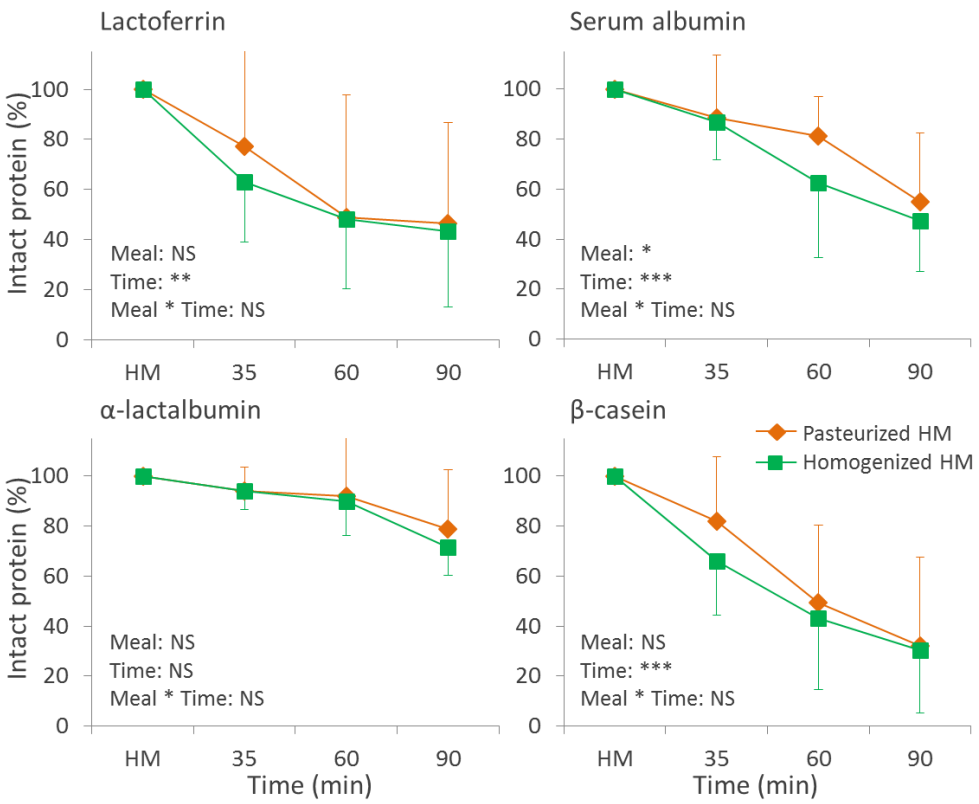
the analyses of human milk macronutrient compositions; Célia Moustiés for her laboratory work and analyses; Mathieu Emily, Florien Kroell, Bruno Laviolle and Alain Renault for statistical advices.

4.5. Supplementary material



Supplementary Figure 6. Flow diagram of infants included in the study. The number of samples potentially collected per modality is indicated. Only one group was formed: each infant was his or her own control to compare the gastric digestion of pasteurized and pasteurized-homogenized human milk (PHM and PHHM).



Supplementary Figure 7. Kinetics of proteolysis of PHM and PHHM during gastric digestion. The percentage of each intact protein remaining in the stomach at a given time was estimated in comparison with the corresponding undigested human milk (HM) and corrected for meal dilution and emptying using taurine (soluble phase) as a meal marker. Determination was done by SDS-PAGE followed by densitometry $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*); $P > 0.05$ (NS). Data from undigested milk were not included in the statistical analysis.

Chapter 5 – Specificity of gastric conditions during digestion of different types of milk: new *in vivo* data from preterm infants

Based on the following projects of publication:

Gastric lipase and other lipolytic activities in the preterm fed raw, pasteurized or pasteurized-homogenized human milk

S.C. De Oliveira[1], Y. Le Gouar[1], O. Ménard[1], D. Faure-Bidégaray[1], A. Bellanger[2], E. Dirson[3], P. Pladys[2], F. Carrière[4], D. Dupont[1], A. Deglaire[1], C. Bourlieu[1*]

[1]Agrocampus Ouest INRA, STLO, Rennes, France; [2]CHU Rennes, Department of Pediatrics, France; [3]CHU Rennes, Lactarium - Infant Nutrition and Dietetics, France; [4]CNRS-Aix-Marseille University-UMR 7282 EIPL, Marseille, France.

Physiological conditions during preterm gastric digestion and considerations for *in vitro* dynamic digestion simulation

S.C. De Oliveira[1], O. Ménard[1], Y. Le Gouar[1], G. Henry[1], A. Bellanger[2], E. Dirson[3], P. Pladys[2], F. Carrière[4], D. Dupont[1], C. Bourlieu[1] A., Deglaire[1*]

[1]Agrocampus Ouest INRA, STLO, Rennes, France; [2]CHU Rennes, Department of Pediatrics, France; [3]CHU Rennes, Lactarium - Infant Nutrition and Dietetics, France; [4]CNRS-Aix-Marseille University-UMR 7282 EIPL, Marseille, France.

Understanding the behavior of HM in the infant gastrointestinal tract is a key step for developing substitutes with health benefits for the neonate. However, ethical and technical reasons limit the possibility of *in vivo* trials. As a consequence, it is important to develop consistent *in vitro* models. Thus, the objective of this chapter is to answer the questions:

Does Holder pasteurization and ultrasonic homogenization impact, in vivo, physiological conditions during gastric digestion in preterm infants?

Can the dynamic in vitro model of digestion DIDGI® be improved based on these new set of data?

In order to answer the first question, section 5.1 presents the characterization of gastric conditions during the digestion of RHM, PHM and PHHM in preterm tube-fed infants: fasting and postprandial lipolytic activities, and fasting pH and gastric volume. These data were collected during the clinical trial presented in chapters 3 and 4 of this manuscript.

Subsequently, section 5.2 compares the evolution of RHM and PHM during *in vitro* and *in vivo* gastric digestion in preterm infants (chapters 2 and 3, respectively). Finally, the set of data from this clinical trial is used for giving new insights for improving the setup of the dynamic *in vitro* model presented in chapter 2.

Both 5.1 and 5.2 sections are independent articles in preparation for submission. They will be further complemented with some data currently being analyzed, *i.e.* postprandial lipase activity in section 5.1 and creaming during gastric emptying in section 5.2.

5.1. Gastric lipase and other lipolytic activities in the preterm fed raw, pasteurized or pasteurized-homogenized human milk

5.1.1. Abstract

Hydrolysis of milk lipids is an essential step in their digestion, initiated in the digestive tract by human gastric lipase (HGL). The aim of this study was to determine HGL activity, output and the contribution of other lipolytic enzymes in gastric aspirates of infants in fasting state or after administration of raw, pasteurized or pasteurized-homogenized human milk (RHM, PHM, PHHM, respectively). *In vivo* study was conducted on preterm infants fed by nasogastric tube (NCT02112331), included in two independent groups determining the type of meals: A) RHM and PHM; B) PHM and PHHM. Lipolytic activity was determined by titration of released free fatty acids from tributyrin (pH-stat technique, at pH 4.5, 6 and 8, at 37°C). Gastric pH and total volume were monitored. The fasting gastric pH was 3.7 ± 1.0 in group A and 4.3 ± 0.6 in group B. The fasting volume was on average 2.5 ± 2.7 and 2.2 ± 2.8 for group A and B, respectively. Gastric lipolytic activity was determined in 20 preterm infants in fasted state, indicating a global HGL concentration of 62.00 ± 0.01 µg/mL and global lipolytic activity of 28.7 ± 4.1 U/mL at pH 4.5. This fasted activity was inversely correlated with pH and gastric volume. Postprandial activity was higher after administration of RHM compared to PHM, disregarding the postprandial time (respectively 17.4 ± 3.6 and 6.9 ± 2.7 U/mL/kg at 90 min, p = 0.010). Homogenization had no effect on postprandial lipolytic activity. The influence of RHM administration suggests that, in addition to conveying additional activity *via* its content in bile salt stimulated lipase, this milk may enhance gastric secretions compared to PHM.

Keywords: digestion; enzyme activity; gastric lipase; human milk; preterm infant.

5.1.2. Introduction

Hydrolysis of milk lipids is an essential step in their digestion, initiated in the digestive tract by human gastric lipase (HGL) (Bakala N'Goma et al., 2012; Aloulou & Carrière, 2008; Sarles, Moreau, & Verger, 1992). Although limited, gastric lipolysis has been presented as a key phenomenon in efficient fat digestion for newborns (Lindquist & Hernell, 2010; Bernback et al., 1989). It compensates for the immaturity of exocrine pancreatic function and favors the subsequent action of other lipolytic enzymes such as human pancreatic lipase or bile salt dependent lipase (HPL and BSSL, respectively) in the intestinal phase.

Some values of HGL activity have been determined in gastric aspirates of infants in fasting or postprandial states (Roman et al., 2007; Armand et al., 1996; Armand, Hamosh, Mehta, & Angelus, 1994; Hamosh et al., 1981; Hamosh et al., 1978; Friedrikzon & Hernell, 1977). High variability was

reported probably due to the influence of the infant age, the time after the last feeding or the analysis method.

In these studies, the HGL output has never been estimated after administration of various types of human milk, nor the potential contribution of other lipases than HGL to the global gastric lipolytic activity. Indeed, in addition to HGL, can be present in the stomach the BSSL (carried by raw human milk) or HPL (in case of duodenal reflux). The curves of lipolytic activities against pH for these three putative lipases present in gastric aspirate are presented in **Figure 36**, suggesting that these enzymes could be actives in the gastric phase (Bourlieu et al., 2014; Bakala N'Goma et al., 2012).

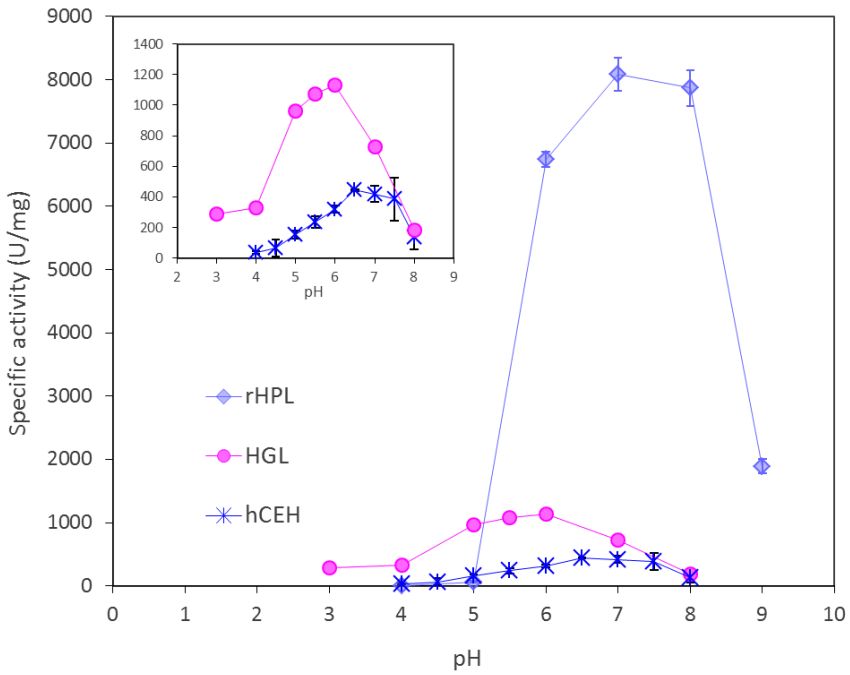


Figure 36. Curve of specific activity of HPL, HGL and human cholesterol ester hydrolase (hCEH), considered as analogue to BSSL, against pH. Recombinant Human pancreatic lipase (rHPL) was measured in the presence of colipase on tributyrin (0.3 mM Tris, 150 mM NaCl, 2 mM CaCl2, 4 mM NaTDC) at 37 °C (Erlanson & Borgstrom, 1970). HGL specific activity was measured as specified in the present paper and data withdrawn from Gargouri et al. (1986). Native human hCEH specific activity was measured by titration with assay solution based on 1 mM Tris–HCl buffer, 150 mM NaCl, 1.4 mM CaCl2, and 4 mM sodium taurodeoxycholate (Personnal data from F. Carrière on hCEH).

The objective of the present study was thus to determine HGL activity and output in gastric aspirates of preterm infants in fasting state or after administration of RHM, PHM or PHHM. In addition, fasting state contents were characterized in terms of pH, total volume and the contribution of other lipases to the global gastric lipolytic activity. The activity of endogenous BSSL in human milk samples was assessed.

5.1.3. Materials and methods

In vivo study and digesta sampling

In vivo study was conducted at Rennes Hospital on preterm infants (< 32 SA) fed by nasogastric tube every three hours (NCT02112331), as detailed in De Oliveira et al. (2017a; 2017b). This clinical trial was

approved by the Institutional Review Board of University Hospital Center of Nantes (CPP Ouest IV), France, and parents gave informed written consent. The infants were included in two independent groups determining the type of meals: A) RHM and PHM, n = 12; B) PHM and PHHM, n = 8. Each infant received the two types of milk every day over a six-day sequence (20 mL/kg of body weight); the other six out of eight daily meals were not controlled. Postprandial digesta were collected at 35, 60 or 90 min after administrated meal. Fasted gastric contents were collected three hours after last meal (up to 12 times per patient to verify intra-individual variation). After collection, aspirates were immediately blended with glycerol (50:50 v/v) and 10 µl of a solution of Pepstatin A at 0.72 M (Sigma Aldrich, St-Quentin Fallavier, France) per mL of sample and frozen at -20 °C. For each patient, two fasting aliquots were randomly selected over the six-day sequence during which the clinical trial was conducted. Gastric volume and pH decrease were monitored.

Human milk collection

Preterm human milk samples were obtained either directly from the mother as fresh (group A) or from the donor milk bank of the University Hospital Center in Rennes (France) after Holder pasteurization only or pasteurization and ultrasonic homogenization (group A and B). The conditions of collection, storage, pool and pasteurization of preterm human milk were previously detailed by de Oliveira et al. (2017a; 2017b). All milks were stored at -20 °C until analyses.

Lipolytic measurement of activity by pH-stat

Lipolytic activity was assessed in a thermostated vessel (37 °C ± 0.5 °C) by pH-stat (Titrando 842, Metrohm SA, Courtaboeuf, France) using tributyrin as substrate, as detailed by Gargouri et al. (1986). Briefly, the assay solution was composed by 2 mM sodium taurodeoxycholate, 150 mM NaCl and bovine serum albumin (0.1 g/L), with adjustment of pH according to the pH of measurement 4.5. An amount of 0.8 mL of substrate (tributyrin) was dispersed in 24 mL of assay solution. The quantity of fatty acid (FA) released upon tributyrin hydrolysis was titrated using 0.1 N of NaOH and corrected taken into consideration a theoretical pka of butyric acid of 4.75. An amount of 25 to 100 µL of the gastric aspirate was used for activity determination, paying attention to minimize the volume tested to always remain in large excess of substrate. The lipolytc activity was expressed as enzyme units per mL of gastric content (U/mL), with 1 U corresponding to the release of 1 µmol of butyric acid per minute under the assay conditions. Each aliquot was measured at least in triplicate.

Lipolytic activity in undigested human milk was determined by titration as described for gastric aspirates. Measure was performed at pH 8 according to the method described for BSSL and HPL [13]. Briefly, the assay solution was this time based on 1 mM tris(hydroxymethyl)aminomethane, 150 mM Nacl, 5 mM $CaCl_2$ and 4 mM sodium taurodeoxycholate.

Calculation of lipase contribution

Subsequent determinations of lipolytic activity at pH 6 and pH 8 allowed the estimation of non-gastric lipases contribution to the global activity (**Figure 36**) in fasted samples. Thus, determining the lipolytic activity at pH 4.5, 6 and 8 allowed setting and solving the following system of equations:

$$\begin{cases} Activity_{pH4.5} = 643.8X + 65Y \\ Activity_{pH6} = 1130X + 320Y + 6743Z \\ Activity_{pH8} = 183X + 135Y + 7872Z \end{cases} \quad (1)$$

with X, Y and Z the quantity in mg of, respectively, HGL, BSSL and HPL in the gastric aspirate and the coefficients corresponding to the specific activity (U/mg) of the pure lipase at the given pH of the measurement. For instance, at pH 4.5 the specific activity of HGL is 643.8 U/mg of enzyme, that of BSSL is 65 U/mg, and HPL has no activity at this pH. The system of equations was solved by substitution.

Statistical analysis

Results were expressed as mean ± SEM for enzyme activity and mean ± SD for pH and gastric volume. Differences were analyzed by unpaired T-test. A p-value < 0.05 was considered as statistically significant.

5.1.4. Results

Fasting state characterization and individual lipases contribution

The fasting gastric pH was 3.7 ± 1.0 (range: 1.7 to 5.6) in group A and 4.3 ± 0.6 (range: 2.6 to 5.2) in group B (**Figure 37**A). Concerning the fasting gastric volume, it was 1.5 ± 1.6 mL/kg of body weight (range: 0 to 8.4) in group A and 1.3 ± 1.7 mL/kg of body weight (range: 0 to 9.7) in group B (**Figure 37**B). The total fasting volume was on average 2.5 ± 2.7 and 2.2 ± 2.8 for group A and B, respectively.

The results of fasting activities measured at pH 4.5, 6 and 8 for each group are represented in **Figure 38**. In group A, activity at pH 4.5 presented a mean value of 15.9 ± 3.8 U/mL/kg, which corresponds to a global gastric content of 80.4 ± 16.2 U/kg. Similar activity was detected at pH 6 with a mean value of 17.9 ± 3.3 U/mL/kg, which corresponds to a global gastric content of 90.8 ± 9.2 U/kg. Activity determined at pH 8 was quasi-null in most aliquots (on average 3.8 ± 1.6 U/mL/kg). For 11 out of 14 measures, values ranged from 0 to 3.2 U/mL/kg, indicating the absence of HPL in these aliquots. The three other measures at pH 8 corresponded to 10.1, 13.5 and 17.5 U/mL/kg. The mean amount of HPL in these three samples, as determined by the system of equations (1), was 3.7 ± 1.6 µg/mL.

In group B, similar trend was observed with an activity at pH 4.5 of 19.9 ± 5.1 U/mL/kg and a global content in the stomach of 78.7 ± 28.6 U/kg. Again, close values were obtained at pH 6: activity of 16.1 ± 2.7 U/mL/kg and global activity of 58.2 ± 14.9 U/kg. In this group, no aliquot presented activity at pH 8.

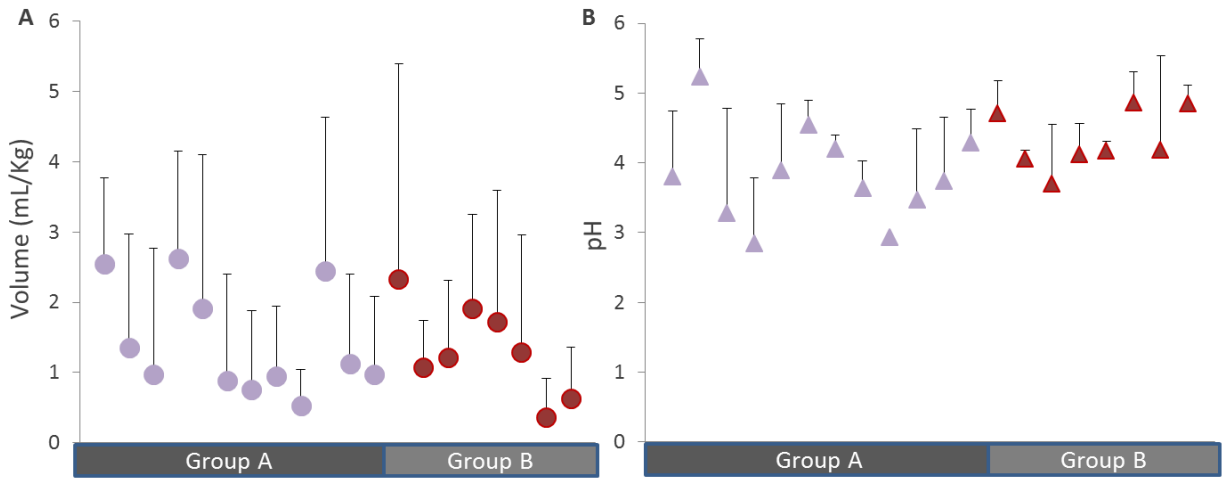


Figure 37. Fasting gastric pH and total volume in preterm infants fed every three hours. Group A (n= 12), group B (n = 8)

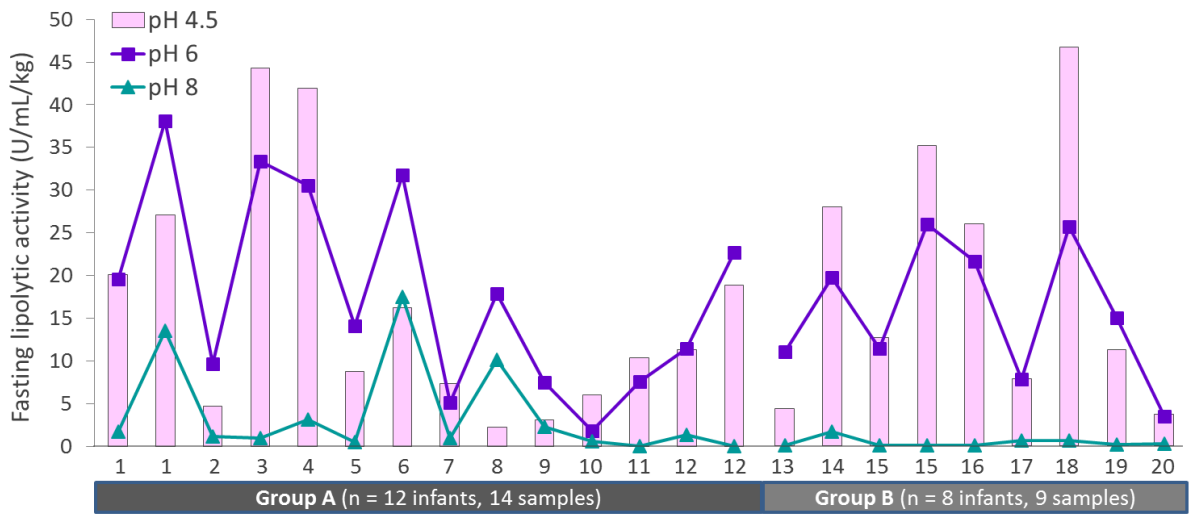


Figure 38. Fasting lipolytic activities in patients of group A (n= 12) and B (n = 8) measured at pH 4.5, 6 and 8. Measurements were conducted in triplicate on one aliquot.

HGL was dominant in fasting state and present at the same concentration in groups A and B (respectively 61.1 ± 13.7 µg/mL and 68.9 ± 19.0 µg/mL, p = 0.5).

Fasting lipolytic activities against total volume or pH were presented in **Figure 39**. Whatever the group, at pH 4.5 and 6 the fasting lipolytic activities were inversely correlated with total volume and pH.

The global activity obtained in our clinical study was confronted to previous results of the literature in **Table 14**.

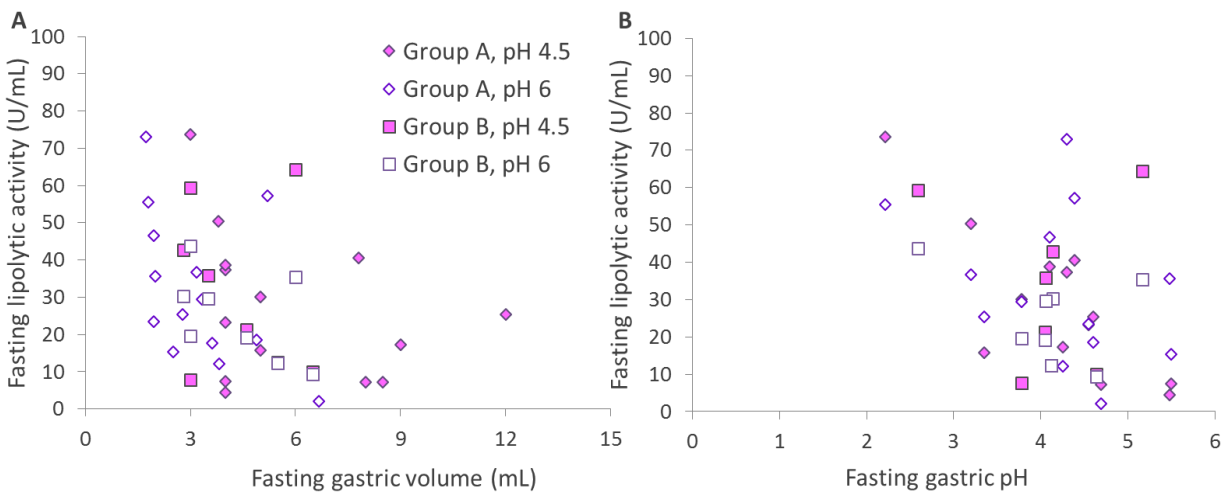


Figure 39. Fasting lipolytic activities at pH 4.5 and 6 against gastric volume A) and pH B). Data point represents triplicate determination by pH-stat.

Lipolytic activity in human milk

Average activity determined on RHM was 77.6 ± 17.2 U/mL (n = 4), whereas no activity was determined on PHM. PHHM was not tested but considered with null activity since homogenization was conducted in indirect mode after pasteurization.

Postprandial activities and influence of meal

Postprandial activities were displayed in **Figure 40**. Infants from group A (n = 4) presented significantly higher activity after RHM ingestion compared to PHM, disregarding the postprandial time. On average, values in U/mL/kg were 11.4 ± 3.3 *versus* 2.9 ± 1.0 at 35 min (p = 0.007), 17.2 ± 5.8 *versus* 5.1 ± 1.5 at 60 min (p = 0.019), 17.4 ± 3.6 *versus* 6.9 ± 2.7 at 90 min (p = 0.010) respectively for infants fed RHM *versus* PHM. Time did not impact significantly lipolytic activity in group A whatever the meal, though a tendency was observed in infant fed PHM. In group B, the effect of time was significant but homogenization of PHM did not impact lipolytic activity, with average values, in U/mL/kg, of 4.2 ± 0.6 at 35 min, 10.1 ± 2.5 at 60 min and 12.1 ± 2.7 at 90 min.

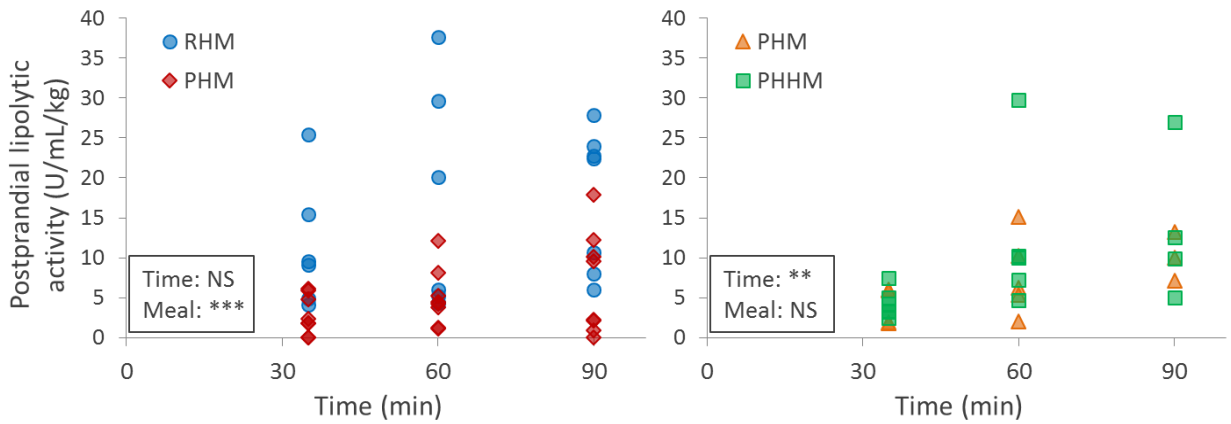


Figure 40. Postprandial lipolytic activities at pH 4.5 for group A, n = 4, and group B, n = 4. For each patient and each type of meal, measurement was conducted on two distinct samples corresponding to the repetition of the postprandial time over the six-day sequence. Data were analyzed by unpaired T-test.

Table 14. Comparison of the values of lipase activity determined in fasting state in gastric aspirates of healthy full-term or preterm infants in the present and previous studies. Adapted from Bourlieu et al. (2014).

Group: N; GA; Age	Birth weight (kg)	pH of collected sample	Gastric activity (U/mL)	References	Additional information (collection time, assay)
N = 7; 38-41 wk; 2.1 ± 1.9 d	3.43 ± 0.51	2.5 ± 0.5	38.9 ± 22.8	(Friedrikzon and Hernell, 1977)	Collection before the first meal (N=4) – activity against p-nitrophenyl acetate determined by spectrophotomety at pH 5.5;
N = 3; 33-36 wk; 27 ± 9.2 d	1.94 ± 0.22	3.0 ± 0.7	18 ± 13.6		Collection in fasting subjects who had already been fed – similar assay as above
N = 9; 27 ± 0.5 wk ; n.a.*	0.83 ± 0.03	5.56 ± 0.5	0.321 ± 0.117	(Hamosh et al., 1981)	Collection at birth – [tri^{3}H]oleate as emulsified substrate test at pH 4.2
N = 22; 32 ± 0.4 wk; n.a.	1.30 ± 0.03	5.74 ± 0.3	0.328 ± 0.028		
N = 24; 34 ± 0.4 wk; n.a.	1.77 ± 0.02	5.70 ± 0.19	0.350 ± 0.053		
N = 35; 35 ± 0.2 wk; n.a.	2.25 ± 0.03	6.25 ± 0.12	0.608 ± 0.034		
N = 22; 37 ± 0.3 wk; n.a.	2.76 ± 0.02	5.36 ± 0.30	0.564 ± 0.062		
N = 30; 39 ± 0.2 wk; n.a.	3.32 ± 0.05	4.93 ± 0.17	0.501 ± 0.041		
N = 13; 30-34 wk; n.a.	1.05-1.78	4.0 ± 0.17	0.048 ± 0.051 (0.004 to 0.140)	(Hamosh et al., 1978)	Collection before feeding – doubly labelled ^{3}Hglyceryl-^{14}C tripalmitin – optimal pH of lipase 5.4
N = 11; 28.9 ± 1.4 wk; 5.5-7.5 wk	1.18 ± 0.07	3.2 ± 0.3	7.9 ± 0.8	(Armand et al., 1996)	Collection before feeding – infant studied 1 to 5 times – [tri^{3}H]oleate as emulsified substrate test at pH 5.4
N = 9; 29.1 ± 0.9 wk; 4.6-7.0 wk	1.09 ± 0.09	3.4 ± 0.5	8.9 ± 1.8		
N = 8; 28.9 ± 1.4 wk; 5.8-7.4 wk	1.00 ± 0.14	3.4 ± 0.3	6.8 ± 0.9		
N = 9; 29 ± 1 wk; 5.3 ± 1.8 wk	1.53 ± 0.55	7.0-3.35 ± 0.87	Close to zero	(Roman et al., 2007)	Collection twice a day for 5 days before feeding – tributyrate substrate test using pH-stat
N=12; 30.0 ± 1.1 wk; 27 ± 12 d	1.37 ± 0.29	4.2 ± 0.3	27.1 ± 5.1 (pH 4.5) 32.1 ± 5.8 (pH 6) 31.1 ± 7.4	Present study	Collection duplicated over a six-day period, at least 3h after last meal and before feeding next one; tributyrate

* n.a.: non available
GA, gestational age.

5.1.5. Discussion

Values in fasted state are in the range with the ones reported by Friedrikzon and Hernell (1977), but much higher than the ones reported by Hamosh et al. (1981; 1978). The low activities reported by the last authors may be explained by collection at birth and before first feeding, but are however in contradiction with the well-established fact that even preterm infants have a mature secretion of gastric lipase. Variation in reported fasting activities is quite high and can explain the wide range of values reported in **Table 14** (Roman et al., 2007; Armand et al., 1996). Our values were inversely correlated with fasting pH and volume, indicating that when the gastric content was not diluted by residual meal, higher lipolytic activity was detected in the stomach; this trend seems to indicate that the contribution from BSSL carried by residual human milk was minor in fasting state. Friedrikzon and Hernell (1977) did not observe any correlation between activity in fasted state and pH, nor with the clinical conditions of the infant, neither with its gestational age, nor postnatal age nor birth weight. However, these authors concluded that test meal stimulated lipase secretion, but they did not check whether different types of milk could trigger different levels of gastric lipase secretion.

The postprandial values reported here are instructive since they suggest that the type of meal influence gastric lipase secretion. Indeed, RHM triggered higher lipolytic activity compared to PHM, although this need to be confirmed in a larger sample of subjects. In adult also, it has been shown that the type of meal and specifically high fat diet enhanced HGL secretion (Armand et al., 1995). Average values at pH 4.5 after RHM administration were of 25.8 U/mL at 60 min, which is close to those reported by Friedrikzon and Hernell (1977) with values of 2 to 20 U/mL, or by Roman et al. (2007), with values of 11.4, 10.7 and 22.8 U/mL at 30, 60 and 90 min, respectively, after administration of an infant formula.

The potential contribution of BSSL in these gastric contents in the presence of low amounts of biliary salts, both brought by RHM, can be questioned. Indeed, from 0 to 30 min, infants ingested 20 mL/kg of human milk, which brings an activity of 77.6 U/mL as measured at pH 8. Taking into account the specific curve of the activity of BSSL (**Figure 36**), it means that 37.4 U/mL of BSSL at pH 4.5 were conveyed by the meal if we consider that milk is not emptied nor diluted in the gastric compartment. However, De Oliveira et al. (2017a) established that gastric content had been emptied at 65% and partially diluted by gastric fluid at 35 min after administration of RHM or PHM. Thus, even if it cannot be excluded that BSSL contributed to the higher activity observed at 35 min in infants fed RHM compared to PHM, HGL seems to be predominantly at the origin of this activity. Indeed, lipolytic activity after RHM-feeding tended to be stable over digestion whereas meal carried on emptying. Furthermore, the gap in lipolytic activity between RHM and PMH-fed infants persisted, which seems

to indicate a direct contribution of gastric secretion to this difference between the two types of milk. Total lipase activity in the gastric compartment was of 163.5 U/kg at 35 min and 234.5 U/kg at 60 min after RHM administration whereas, 42.1 and 62.1 U/Kg were encountered for the same postprandial times after PHM feeding. In spite of these differences, the gastric lipolysis degree were similar for the two meals (de Oliveira et al., 2017a).

Homogenization, on the contrary, did not modify gastric lipolytic activity as compared to PHM (**Figure 40**B). Since homogenization significantly slowed down gastric emptying (de Oliveira et al., 2017b), lipase activity in terms of total unit in the gastric volume is higher than for PHM, with 97.8 *versus* 38.5 U/kg ($p = 0.02$), respectively.

In this study, we demonstrated that gastric lipolytic activity was, on average, three to four times higher after administration of RHM compared to PHM. However, this higher activity did not enhance the gastric lipolysis level from 35 to 90 min. We can thus wonder the physiological role of this higher lipolytic equipment: does it help gastric lipolysis on the very short time, which could not be estimated in our clinical study? Or does this higher HGL output towards the duodenum further facilitate intestinal lipolysis and fat uptake? Collecting faeces and determining coefficient of fat absorption in group A could have helped solving this question but it was beyond the scope of our study.

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5.2. Physiological conditions during preterm gastric digestion and considerations for *in vitro* dynamic digestion simulation

5.2.1. Abstract

To develop relevant *in vitro* models is crucial for studying infant digestion, but input parameters must be established with caution. The purpose of this study was to discuss the recent characterization of fasting and postprandial gastric contents of preterm infants in order to improve our *in vitro* dynamic model simulating newborn digestion. Overall, gastric behaviors of preterm raw and pasteurized human milk (RHM and PHM) as evaluated *in vitro* and *in vivo* were well correlated, with no impact of pasteurization on gastric lipolysis, selective proteolysis and higher aggregates for RHM compared to PHM. However, some parameters may be adjusted for better mimicking the gastric digestion conditions in preterm newborns, *i.e.* time of ingestion (from 10 to 30 min), pH decrease (by linear or polynomial fitting) and proportion of meal in total volume (residual meal). *In vivo*, the residual meal differed depending on the marker (taurine for soluble phase and total fatty acids for lipid phase). Some creaming occurred during gastric digestion for RHM, PHM and homogenized PHM (PHHM), resulting in faster emptying for the soluble phase. Whether this colloidal behavior of the emulsion was affected by pasteurization or homogenization is currently being analyzed and will be the base for adjusting the secretion output and/or emptying rates in our *in vitro* model. This study presents a unique set of data illustrating the specificity of preterm infant gastric digestive conditions. The proposed model of *in vitro* digestion will be useful to scientists and food manufacturers who focus on neonatal digestion.

Keywords: digestion; gastrointestinal; human milk; *in vitro* model; newborn infant.

5.2.2. Introduction

The digestive functions are immature during the first months of life, especially for preterm newborns (*i.e.* motility, enzymatic and secretory functions) (Bourlieu et al., 2014). The gastric digestion of complex colloidal emulsions such as human milk and infant formulas is a key step which will further modulate nutrient absorption and infant nutrition (Lindquist & Hernell, 2010; Hamosh et al., 1998). Factors such as gastric acidification, emptying rate and enzyme outputs are key parameters that play a role in the kinetics of hydrolysis and the disintegration of these emulsions, and may be influenced by the nature of the meal and the age of the infant (Sams et al., 2016; Bourlieu et al., 2014).

Studying digestion requires the use of *in vitro* or *in vivo* models. *In vivo* studies in children are limited mainly due to obvious ethical concerns, and remain complicated and expensive. *In vitro*, the models do not reproduce the biological complexity of the digestive tract but consist on a simple

implementation option that can provide valuable information when comparing different matrices (Ménard & Dupont, 2014; Guerra et al., 2012). Consequently, it is necessary to develop relevant *in vitro* models, for which the parameters used must be established with caution, depending on the stage and meal to be digested (Bourlieu et al., 2014; Levi & Lesmes, 2014; Shani-Levi et al., 2013).

The purpose of this study was to discuss recent characterization of fasting and postprandial gastric contents of preterm infants in order to improve our *in vitro* dynamic model simulating newborn digestion.

5.2.3. Materials and methods

In vivo study was conducted at Rennes Hospital on preterm infants fed by a nasogastric tube (NCT02112331) (de Oliveira et al., 2017a; de Oliveira et al., 2017b; de Oliveira et al., 2017c). Briefly, newborns were included in two independent groups determining the type of meals: A) raw or pasteurized human milk (RHM or PHM); B) pasteurized or pasteurized-homogenized human milk (PHM or PHHM). Gastric contents were collected twice a day, over six days, before meal ingestion and 35, 60 or 90 min after meal ingestion. Gastric volume and pH were measured. Lipase activity was determined by titration of released free fatty acids from tributyrin (pH-stat technique, at pH 6 and pH 8, at 37°C). Gastric emptying rates and ratio of meal to secretions were determined using two different meal markers: total fatty acids for the lipid phase and taurine for the liquid phase. The markers were quantified respectively by gas and cation-exchange chromatography.

A dynamic *in vitro* digestion system developed by the French National Institute for Agricultural Research (DIDGI®) was set up in terms of types and amounts of enzymes, secretions, pH decrease and emptying rate to mimic as closely as possible the gastrointestinal digestive conditions of term and preterm newborns (de Oliveira et al., 2016; de Oliveira et al., 2015). Gastric preterm *in vitro* digestion, were sampled regularly from 30 to 90 min, was compared with that *in vivo*. Preterm pooled RHM or PHM were digested in triplicate.

In vitro and *in vivo*, structural changes were evaluated by confocal microscopy and laser light scattering; lipolysis and proteolysis kinetics were monitored by SDS-Page, thin-layer and gas chromatography methods.

Data were expressed as mean ± SD.

5.2.4. Results and discussion

Preterm human milk gastric digestion as studied in vitro and in vivo

The impact of pasteurization on the preterm gastric digestion of HM was assessed in both *in vitro* and *in vivo* studies.

Results showed a good correlation between *in vivo* and *in vitro* data concerning the disintegration of milk fat globules as observed by CLSM, which shows that structure of fat globule was maintained during the gastric digestion. The impact of pasteurization on the structure was less pronounced *in vivo*, when strong emulsion destabilization was observed for both RHM and PHM. *In vitro*, this strong emulsion destabilization was observed only for RHM, while PHM did not evolve that much. From these results obtained *in vitro*, it was suggested that this different pattern of emulsion disintegration could affect gastric emptying rate *in vivo*. However, this was not observed *in vivo* (from 35 to 90 min); *in vivo*, pasteurization led to the formation of smaller aggregates, but both RHM and PHM systematically presented strong emulsion destabilization. The more complex environment during *in vivo* compared to *in vitro* digestion is certainly responsible for these differences.

In spite of the inactivation of BSSL by pasteurization (Henderson et al., 1998), the instantaneous lipolysis degree during preterm gastric digestion was not affected ($p > 0.05$). An overall, similar degree of instantaneous lipolysis was found *in vitro* and *in vivo* (respectively 11.3 ± 3.8% and 12.6 ± 4.7 at 90 min).

Pasteurization did not significantly affect the gastric kinetics of proteolysis in the preterm ($p > 0.05$), except *in vivo* for lactoferrin and α-lactalbumin (only at 90 min). In all the digestions, gastric proteolysis appeared to be selective, with greater resistance for α-lactalbumin and faster digestion for lactoferrin and β-casein.

Overall, gastric behaviors of preterm RHM and PHM as evaluated *in vitro* and *in vivo* were well correlated. Whether differences concerning proteolysis are a result of the dissimilarities between models or between the milks is unclear. It is important to note that, as expected, the triplicated *in vitro* digestions presented high repeatability whereas *in vivo* digestion presented high inter-and intra-variability.

Postprandial gastric conditions in preterm infants and considerations for in vitro digestion simulation

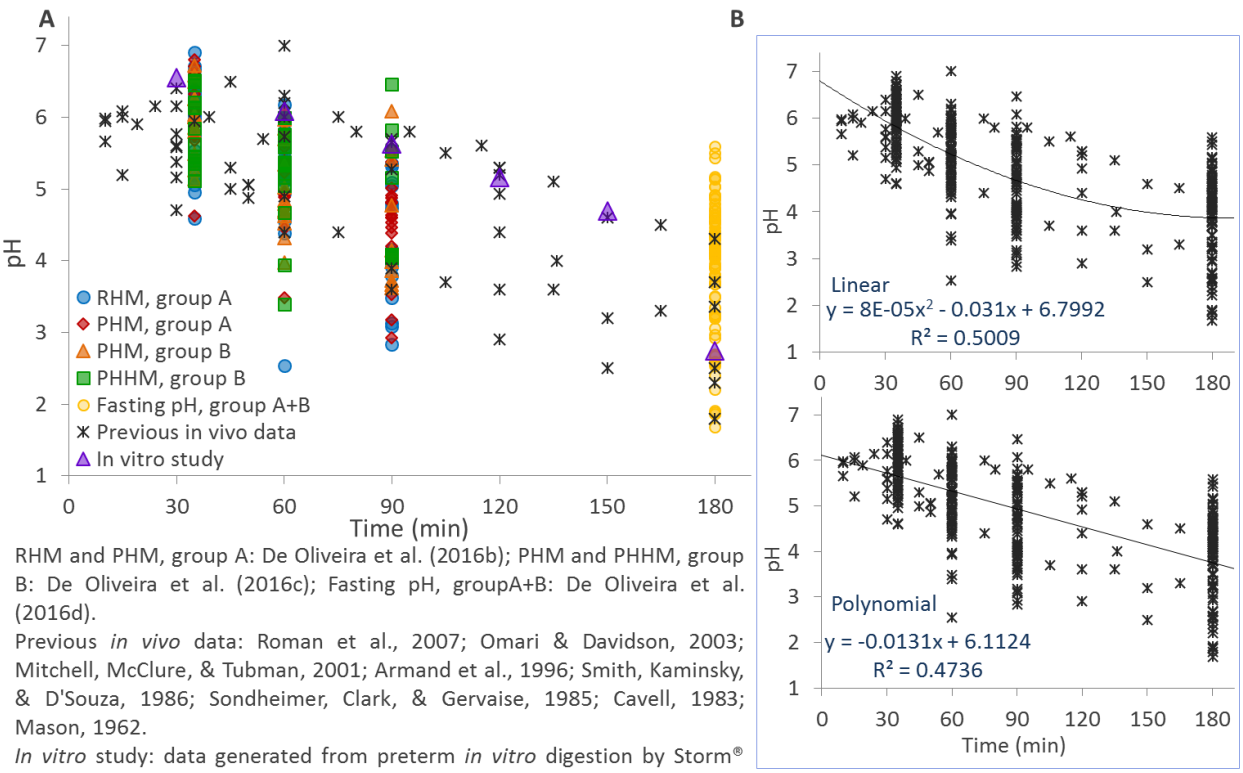
Fasted state

In vitro, the volume for fasting state was set at 2 mL of simulated gastric fluid (SGF) (Geigy, 1973). It was adjusted at pH 2.7, as averaged from reported pH values in fasted state for preterm newborn infants fed every 3 h (Roman et al., 2007; Omari & Davidson, 2003; Mitchell et al., 2001; Armand et al., 1996; Smith et al., 1986; Sondheimer et al., 1985). Considering the totality of the infants from groups A and B, on average the fasting volume and pH were 1.4 ± 1.6 mL/kg (*i.e.* 2.4 ± 2.8 mL) and 3.9 ± 0.9, respectively (de Oliveira et al., 2017c). Thus, we consider that fasting volume at 2 mL is within the same range than that found in the present study. Concerning pH, gathering present and previous

data, new averaged fasting pH is 3.1 ± 0.9, which remains close of the previous averaged pH (2.7). In any case, it seems that this initial pH will be neutralized as soon as the milk-based meal arrives in the stomach.

Gastric acidification

As observed in our study *in vivo*, gastric acidification was not impacted by the type of milk ($p > 0.05$) (de Oliveira et al., 2017a; de Oliveira et al., 2017b). As shown in **Figure 41**A, gastric pH values found in our *in vivo* study were within those reported in the literature and used for the estimating *in vitro* pH decrease (Roman et al., 2007; Omari & Davidson, 2003; Mitchell et al., 2001; Armand et al., 1996; Smith et al., 1986; Sondheimer et al., 1985; Cavell, 1983; Mason, 1962). Altogether, these data confirm the preterm newborn limited capacity of acidification (Bourlieu et al., 2014), but also reflect the high buffering capacity of milk-based meals (Salaun, Mietton, & Gaucheron, 2005).



RHM and PHM, group A: De Oliveira et al. (2016b); PHM and PHHM, group B: De Oliveira et al. (2016c); Fasting pH, groupA+B: De Oliveira et al. (2016d).
Previous *in vivo* data: Roman et al., 2007; Omari & Davidson, 2003; Mitchell, McClure, & Tubman, 2001; Armand et al., 1996; Smith, Kaminsky, & D'Souza, 1986; Sondheimer, Clark, & Gervaise, 1985; Cavell, 1983; Mason, 1962.
In vitro study: data generated from preterm *in vitro* digestion by Storm® software. De Oliveira et al. (2015)

Figure 41. A) Gastric pH decrease as reported in infants fed every three hours in previous and in the present *in vivo* studies, and those used for *in vitro* digestion simulation. B) Proposed fitting for gastric acidification by linear or polynomial curves.

As observed in **Figure 41**A, the average pH values estimated for *in vitro* digestion are in the upper part of the *in vivo* data set based on the present and previous published studies. This occurred because the initial pH of the *in vitro* digestion was set at the meal pH. For better mimicking the gastric acidification during *in vitro* newborn digestion, new equations (linear and polynomial) are proposed from fitting previous and present data, as displayed in **Figure 41**B.

Gastric emptying and meal to total volume ratio

As we previously reported, pasteurization did not affect the gastric emptying rate of RHM, as determined from the liquid meal marker; however, homogenization slowed down the emptying of PHM (**Figure 42**A). The half time of gastric emptying proposed *in vitro* (*i.e.* 36 min) was quite close of that found in the present *in vitro* study (30 min for RHM an PHM, and 38 min for PHHM). Importantly, however, we showed that for all types of milk the gastric emptying did not follow the exponential Elashoff pattern proposed for dynamic *in vitro* models, mainly during the ingestion time. The higher estimated proportion of ingested meal present in the stomach coincided with both the end of the ingestion, *i.e.* 50% and 65.3% at 30 min for PHM and PHHM, respectively. Newborns, mainly preterm, usually receive relatively small volume of meal during a long ingestion time; since the emptying is faster than in older infants (Bourlieu et al., 2014), this simultaneous ingestion and emptying should be taken into account for dynamic *in vitro* models in order to avoid a large proportion of meal arriving in the intestinal compartment sooner than it is physiologically expected. Thus, in order to improve the *in vitro* dynamic model previously proposed for the preterm newborn, we recommend an increase in the ingestion time from 10 to 30 min.

In our *in vitro* model, the residual meal dramatically decreased over the time with meal being emptied (**Figure 42**A). This does not correspond to our *in vivo* data, for which this proportion of meal in total volume slightly decreased or remained constant (as determined by the liquid phase marker taurine), as displayed in **Figure 42**B

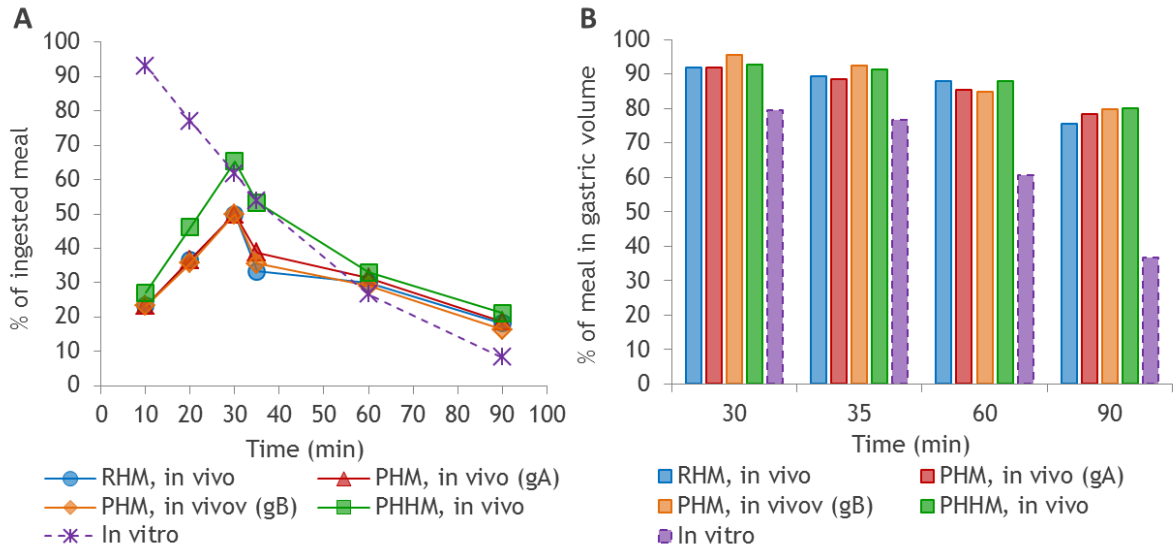


Figure 42. Proportion of the ingested meal present in the stomach (A) and contribution of residual meal to the total gastric contents (B) at a given time after ingestion of raw, pasteurized or pasteurized-homogenized HM (RHM, PHM and PHHM, respectively). *In vitro* data was generated from preterm *in vitro* digestion by Storm® software. RHM and PHM *in vivo* data correspond to that presented in De Oliveira et al. (2017a). PHHM *in vivo* data correspond to that presented in De Oliveira et al. (2017b).

Again, the results presented in **Figure 42** were determined by the liquid phase marker taurine. In our study, the same data was also determined by the lipid phase marker total FA; although the lipid phase corresponds only to ~3% of the milk emulsion, this approach was already used by Roman et al. (2007) and total FA can be easily determined. The combination of these two approaches showed that the proportion of residual meal differed depending on the marker, with some digested samples presenting higher concentration of fat than in the corresponding undigested HM (**Figure 43**). This suggests that creaming occurred during gastric digestion for RHM, PHM and PHHM, resulting in faster emptying for the soluble phase compared to the lipid phase of the emulsion. Whether this colloidal behavior of the emulsion was affected by pasteurization or homogenization is currently being analyzed and will be the base for proposing an adjustment of secretion output and/or emptying rates in our *in vitro* model.

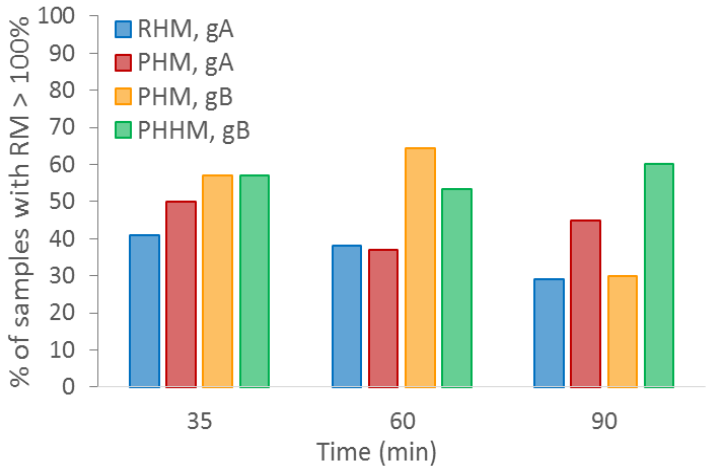


Figure 43. Proportion of samples presenting creaming (digested samples presenting higher concentration of fat than in the corresponding undigested HM) in groups A and B. RM, contribution of the residual meal to the total gastric volume as determined by lipid phase marker (total fatty acids).

5.2.5. Conclusion

This study presents a unique set of data illustrating the specificity of preterm infant gastric digestive conditions. Understanding the physiological environment in the infant gastric tract is a key step for developing physiological relevant *in vitro* models and essential to optimize infant nutrition management. Overall, the proposed setup for the in vitro dynamic model DIDGI was considered pertinent for preterm newborn HM digestion. However, gastric pH acidification, emptying rates and the proportion of meal in total gastric volume could be improved to adjust closer *in vivo* gastric conditions data. The estimation of gastric emptying rates and the proportion of residual meal on the total volume determined by lipid or liquid markers is an original approach and data are currently being analyzed. Finally, the proposed model of *in vitro* digestion will be useful to scientists and food manufacturers who focus on neonatal digestion.

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Chapter 6 – Final considerations

6.1. General discussion

The aim of this thesis was to investigate the impacts of Holder pasteurization and ultrasonic homogenization on the digestion of HM, and more precisely on the kinetics of lipolysis, proteolysis and emulsion disintegration. The impact of pasteurization was firstly investigated *in vitro,* showing that gastric and intestinal phases of digestion were both affected by pasteurization, to a larger extent in term than in preterm stage. This impact was confirmed for the preterm gastric phase *in vivo*. However, contrary to our initial hypothesis, the BSSL inactivation by pasteurization did not affect the gastric lipolysis in preterms. Homogenization, evaluated only *in vivo*, impacted the digestion of PHM mainly by increasing the lipolysis rate and slowing down the gastric emptying.

The originality of our findings consisted mainly in bringing information about the digestive kinetics of different types of HM during gastric digestion, as well as in the small intestine. Indeed, studies in infant nutrition have focused mainly on the impact of different milk-based food on nutritional and health outcomes such as growth, neuronal development, cardiovascular diseases (Victora et al., 2016; Quigley & McGuire, 2014; Rozé et al., 2012; Andersson et al., 2007). Some authors have explained the different impacts by factors such as modulation of microbiota (Bourlieu et al., 2015a; Groer et al., 2014), preprograming mechanisms (Oosting et al., 2014; Oosting et al., 2012) or bioactivity functions exerted by individual components or structure organization specificities (Hernell et al., 2016; Lonnerdal, 2016; Bourlieu & Michalski, 2015; Lonnerdal et al., 2015; Underwood et al., 2015). However, knowledge concerning the digestive behavior of these different types of HM was missing, and our findings bring some insights that may be useful for a more general mechanistic understanding of nutritional and health consequences depending on the food.

The main results from the combination of the *in vitro* and *in vivo* approaches are summarized in **Table 15** and **Table 16**. The impact of technological treatments of HM on the gastric digestive conditions is summarized in **Table 17**. As observed in **Table 15**, the pre-lipolysis degree of undigested HM used in the term *in vitro* study was higher for RHM than for PHM, likely due to the second cycle of freeze-thaw facilitating the action of BSSL in RHM (*cf.* chapter 2). On the contrary, RHM used in the preterm *in vivo* study did not undergo freeze-thaw cycle as it was freshly expressed; its pre-lipolysis degree was lower than that of PHM, likely due to the action of BSSL in PHM during frozen storage, before this batch of PHM was pasteurized and hence the BSSL inactivated. Regarding the undigested HM used in the preterm *in vitro* study, the pre-lipolysis degree did not significantly differ between RHM and PHM, but this can be linked to a shorter storage time. Altogether, these findings show that

pre-lipolysis by endogenous BSSL in HM do take place, and its extent depends on the conditions of processing and storage, probably as important as BSSL levels.

Concerning the gastric kinetics of hydrolysis, results from *in vivo* and *in vitro* studies showed no impact of pasteurization on the gastric lipolysis in the preterm; on the contrary, pasteurization reduced the gastric lipolysis of term HM, which was likely explained by the initial difference in the pre-lipolysis level between RHM and PHM. Disregarding the gastric lipolysis degree, for the two modalities the intestinal lipolysis was enhanced in RHM compared to PHM, which was probably linked to the action of BSSL in RHM. The impact of pasteurization on proteolysis varied, but a systematic selective protein digestion was observed for both RHM and PHM, in particular with greater resistance for α-lactalbumin and faster proteolysis for lactoferrin and β-casein. Complementary to this information, *in vitro* intestinal digestion evidenced that bioaccessibility of AA was selectively modulated by pasteurization.

A common result for term and preterm HM was that Holder pasteurization induced protein aggregation around the MFG membrane. Concerning the emulsion disintegration during gastric digestion, we observed an obvious difference *in vitro*, with strong emulsion destabilization only for RHM. *In vivo*, this destabilization was systematically observed for RHM and PHM, even if the size of the aggregates was, on average, higher for RHM. The more complex environment during *in vivo* compared to *in vitro* digestion is certainly responsible for these differences, *i.e.* the presence of endogenous glycosylated proteins such as mucins, which may modulate the physical behavior of the emulsions. Another explanation could be the higher pH during *in vitro* compared to *in vivo* digestion (*cf.* chapter 5). However, preliminary assays following the turbidimetry of HM during static gastric digestion using a Turbiscan® (Formulaction, L'Union, France) demonstrated that the presence of enzymes determined the emulsion destabilization, but not the pH at 5.2, as measured for 60 min of digestion (data not shown).

As presented in **Table 16**, homogenization of PHM enhanced the gastric lipolysis degree but did not affect proteolysis. Furthermore, the dispersed lipid structure obtained after homogenization persisted during gastric phase, whereas native MFG persisted during PHM gastric digestion.

The gastric conditions considered in our study (**Table 17**) were little affected by the type of HM, except for RHM probably inducing more HGL secretion than PHM, and gastric emptying rate slowing down after ingestion of PHHM compared to PHM. Nevertheless, a possible difference in the creaming patterns of the gastric digesta may have occurred depending on the physical treatments applied to HM (*cf.* chapter 5), which will be further evaluated.

Table 15. The Holder pasteurization of HM impacts its gastrointestinal digestion (term and preterm stages)

	Term	Preterm
Human milk		
Composition	No impact on macronutrient composition, total FA[1] and AA profiles	
Pre-lipolysis	Lower in PHM than in RHM[2]	*In vivo:* Higher in PHM than in RHM *In vitro:* No impact
Structure	Heat-induced protein aggregation around the MFG and in the soluble phase	
Gastric digestion (in vitro and in vivo)		
Lipolysis	Lower in PHM than in RHM	*In vivo* and *in vitro*: No impact on lipolysis degree *In vitro*: release of some FA lower for PHM than RHM (mainly C8:0 to C14:1 c9 and C18:2 c9,c12)
Proteolysis	Slower for β-casein and faster for lactoferrin in PHM than in RHM	*In vivo*: Slower for α-lactalbumin (only at 90 min) and faster for lactoferrin in PHM than in RHM *In vitro*: No impact
Structure	Larger aggregates for RHM and strong destabilization from 60 min	*In vivo*: larger aggregates for RHM at 35 and 90 min, strong destabilization for both RHM and PHM from 35 min. *In vitro*: larger aggregates and strong destabilization for RHM from 30 min; structure quite stable for PHM.
Intestinal digestion (in vitro)		
Lipolysis	Lower in PHM than in RHM (both lipolysis degree and FA release)	
Proteolysis (AA bioaccessibility)	PHM lower than RHM: proline, methionine, glycine, glutamic acid. PHM higher than RHM: tyrosine, phenylalanine, isoleucine.	PHM lower than RHM: proline, serine. PHM higher than RHM: tyrosine, phenylalanine, arginine, α-amino nitrogen.
Structure	Larger aggregates for PHM than for RHM	

[1]The difference in some FA contents between RHM and PHM from the *in vivo* study was not considered as resulting from an impact of pasteurization since the HM was not the same.

[2]The pool of RHM used in the *in vitro* study underwent to two freeze-thaw cycles, which do not correspond to habitual practices and likely accelerated the pre-lipolysis process (*cf.* chapter 2).

Table 16. The ultrasonic homogenization of pasteurized HM impacts its gastric digestion (*in vivo* preterm stage)

Human milk	
Composition	No impact on macronutrient composition and FA profiles
Pre-lipolysis	No impact
Structure	Fragmentation of the native MFG in submicronic droplets with bimodal distribution of the particle size, resulting in a six-fold increase in the specific surface area
Gastric digestion (in vivo)	
Lipolysis	Higher for PHHM than for PHM; no impact on FA bioaccessibility at 90 min
Proteolysis	Faster for serum albumin in PHHM than in PHM
Structure	No impact on size parameters but type of formed aggregates different, depending on the structure of the undigested emulsion (native MFG *vs* blend of submicronic droplets)

Table 17. Impact of physical treatments on the chyme characteristics during the digestion of HM

Holder pasteurization applied to raw HM	
pH decrease	No impact
Postprandial gastric volume	No impact
Gastric emptying rate	No impact
Half-emptying time	30 min for both RHM and PHM
Ultrasonic homogenization applied to pasteurized HM	
pH decrease	No impact
Postprandial gastric volume	Higher for PHHM than for PHM (only at 35 min)
Gastric emptying rate	Slower for PHHM than for PHM
Half-emptying time	30 min for PHM and 38 min for HPHM

The **main strength of this work** is the combination of ***in vitro* and *in vivo* studies** focusing on the infant digestion, which resulted in an original set of data. Remarkably, this combination i) allowed the comparison between results from *in vitro* and *in vivo* preterm gastric digestion, ii) compensated for the lack of access to the intestinal phase *in vivo* by intestinal *in vitro* data, iii) gave new insights for improving gastric *in vitro* models for digestion of the three types of milk.

Being more specific, other **original points concerning the strategy of this thesis were**:

- The use of a set of multi-scale techniques, from the macroscopic to the molecular level, to characterize samples both quantitatively (hydrolysis degree, particle size distribution) and

qualitatively (type of FA, AA, emulsion images). Furthermore, the matrices characteristics were taken into account to assess their influence on further digestion behavior (*i.e.* macronutrient composition, microstructure organization, pre-lipolysis level);

- The *in vitro* dynamic digestion system used is an original homemade tool (DIDGI®, INRA, France) that mimics the gastrointestinal digestion process more realistically than other simplified static or semi-dynamic *in vitro* models, since it simulates pH variations, emptying rates and secretion flows;
- It was the first time that parameters for an *in vitro* dynamic digestion model were proposed for newborn infants digesting HM, while taking into account the digestive specificities of term and preterm newborns;
- We implemented a randomized clinical trial to study, *in vivo*, the digestive fate of HM either raw or processed HM;
- Comparing the digestive behaviors of raw *versus* pasteurized HM brought new insights about the contribution of BSSL to fat digestion and the impact of its inactivation by pasteurization;

Some challenges faced during the thesis are pointed below.

In vitro study: the first challenge was to define the *in vitro* dynamic parameters that closely mimic the digestive conditions found in term and preterm newborns, mainly because *in vivo* data concerning the intestinal phase, term infant stage and pasteurized HM digestion are scarce or missing. The choices were made based on an extensive review about the gastrointestinal conditions of infant digestion from birth to six months of age (Bourlieu et al., 2014), and parameters were adapted following specific characteristics of one-month old infants. Then, came the choice of digestive enzymes, when commercial animal analogues were generally selected. However, commercially available gastric lipases present poor analogy with HGL (*cf.* chapter 1, section 1.4.6). This problem was overcome by using a rabbit gastric extract (RGE) obtained thanks to the collaboration with the EIPL laboratory (CNRS, Marseille).

In vivo study: the clinical trial realized in collaboration with the University Hospital Center of Rennes was a large project that required work at different levels, from the implementation of the project and recruitment of newborns up to the sample analyzes. Initially expected to take one year, the infant recruitment was extended to two years in order to try to obtain 12 evaluable infants in each study. While 12 infants were recruited in group A (milk from the own mother), only 8 were recruited in group B (milk from anonymous donor). Recruitment for group B was difficult since infants' mothers were not supposed to breastfeed or express milk, while most preterm infants in this hospital received HM from their own mother, at least partially.

Sampling: a common challenge for *in vitro* and *in vivo* studies was to collect representative samples of the digestive contents at a given time. Indeed, *in vitro* digested samples and *in vivo* effluents are

complex fluids, where macronutrients can be digested or interact with other molecules even after collection; precautions were taken with addition of enzyme inhibitors to prevent samples of further modifications. Moreover, emulsion destabilization can lead to phase separation. Thus, precautions were taken in order to avoid a non-representative sampling: continuous mixing all over the *in vitro* digestion and collection of the whole gastric effluents *in vivo,* always with gentle mixing before sampling. *In vivo*, misplaced nasogastric tube could lead to questionable sampling or to failure in sampling; nasogastric tube placement was verified by auscultation, accordingly to the routine practice in the service.

Analytical limitations:

Some practical procedures concerning the management of samples were easily followed during the *in vitro* studies realized in the laboratory, but were more difficult during the *in vivo* study in terms of transportation, availability of instruments and limited staff. For instance, some analyses were not performed for all the infants, *i.e.* structural characterization of emulsions and pre-lipolysis quantification of undigested HM (du to difficult direct lipid extraction at the hospital).

The **SDS-PAGE** electrophoresis gives a good image of the proteins disappearance and can be semi-quantitative when followed by densitometry analysis. Due to the impossibility of simultaneously overcoming saturation of the signal from the most concentrated proteins and quantifying the minor ones, only the four major proteins could be analyzed. The signal from κ-casein was also intense when measured in HM; however, we noticed that during digestion this signal was increasing in spite of the meal emptying (data not shown), probably as a result of large peptides coming from the proteolysis of proteins of higher molecular weight (*e.g.* β-casein and lactoferrin).

6.2. General conclusion and perspectives

In summary, the present study highlighted some specificities of the HM digestive behavior and positively answered the question: does processing human milk impact its digestion in newborn infants? Indeed, Holder pasteurization and ultrasonic homogenization of HM affected this digestive behavior in different aspects, which was demonstrated and discussed over the manuscript. This research contributed to a better understanding of the impact of BSSL inactivation on the HM digestion, as well as the role of the initial structure on their digestive fate. The *in vivo* data discussed here are crucial for a better understanding of preterm infant digestive behavior and may help to improve the setup of *in vitro* gastric digestion models. In fact, our proposed protocol for dynamic term and preterm *in vitro* digestion could be adjusted thanks to the *in vivo* trial. More generally, the present model can be used for a better understanding of IF digestion as compared to HM, and could help to improve IF in terms of structure and composition in the aim to better mimic HM digestive

behavior. Finally, our findings provide new knowledge on the infant newborn digestion and may be useful to support the challenging nutritional management of this group, particularly when breastfeeding is unavailable.

From our results, new questions appear necessary to be raised. Thus, some perspectives for further research are proposed bellow.

6.2.1. Short-term perspectives

- The peptide released during all the digestions presented in this thesis (*in vitro* and *in vivo*) were analyzed in order to evaluate the impact of the treatments of HM and better predict potential nutritional impacts of these treatments. Peptides were analyzed by tandem mass spectrometry coupled to liquid nano-chromatography. Data regarding *in vitro* digestion in term newborns have been published in the journal Electrophoresis and showed that pasteurization modified the kinetics of release for half of peptides; furthermore, pre-proteolysis was observed particularly in pasteurized HM, potentially through plasmin system activation. Data generated for other modalities are currently being analyzed.
- We demonstrated, for a group of four infants, that gastric lipolytic activity was higher after administration of RHM compared to PHM (chapter 5, section 5.1). Analyses are currently being performed in order to confirm such results in a larger group. In addition, the lipolytic activity in undigested HM have been measured; a possible link with the pre-lipolysis degree will be evaluated.
- As observed *in vivo*, the gastric emptying of the emulsions appeared not homogeneous, with a trend to cream and destabilize from the earlier times of digestion onwards. The meal emptying was determined by both soluble and lipid phase markers, which represents precious understanding of the physiological environment during gastric digestion and the disintegration patterns of each emulsion analyzed. These data are currently being analyzed and will help to estimate more accurately the meal fraction remaining in the stomach at a given time, and hence the ratio meal to secretion and to enzymes.

6.2.2. Long-term perspectives

Understanding characteristics of HM which could impact on its digestion

In the present study, we took into consideration the chemical composition and the structural aspects of undigested HM for evaluating its digestion as raw material or after technological treatments. Nevertheless, some other points concerning the HM characteristics remain to be clarified in order to have an overall understanding about the factors governing digestion.

Pre-digestion during processing and storage: it is well established that endogenous enzymes of HM can start its digestion, and that a cycle of freeze-thaw may facilitate this pre-digestion. However, how the time of storage at -20 °C in HMB (up to 4 months before pasteurization and 6 after) could influence aspects such as hydrolysis and oxidation is not clear. The evolution of products of hydrolysis, of oxidation and enzyme activity should be evaluated over time during storage of HM either raw (before pasteurization), pasteurized (after thawing and pasteurizing) and pasteurized-homogenized. This knowledge is missing for a more precise control of the storage process in HMB and for evaluating the security of providing homogenized HM after long times of storage.

Moreover, pre-proteolysis and protease activities in HM were not directly investigated in the present study. However, it seems important to understand how the heat treatment could impact the plasmin system mechanisms in HM; development of accurate methods to quantify endogenous milk proteases activities would be necessary.

BSSL activity and bile salt contents: beyond their role on the pre-digestion of HM, BSSL and bile salts reportedly play an essential role in the intestinal lipolysis and fat absorption in newborn. How the variation of these components in HM could affect gastric and intestinal digestion is not clear. To clarify this point, a suitable approach could be using the *in vitro* dynamic model for digesting different types of milk, supplemented with different physiological amounts of bile salts for comparison of nutrients bioaccessibility and disintegration patterns. The coefficient of fat absorption depending on the bile salts amount could be assessed by incubating the bioaccessible fractions obtained after *in vitro* digestion in Caco2 cells.

Structural aspects: specific characterization of the impact of Holder pasteurization and subsequent ultrasonic homogenization on the structure of HM is lacking, mainly considering the quality of the emulsion interface. This characterization, alongside with the one of gastrointestinal emulsion behavior, could help to better understand the influence of initial matrices on the diffusion and adsorption of enzymes.

New insights for improving the nutritional care of infants fed banked-human milk or infant formula

In the present study, BSSL was involved in the pre-lipolysis of RHM and PHM in a slightly larger extent for PHM, but then this contribution was probably caught up during the beginning of the gastric phase for RHM. Contrary to our initial hypothesis, the BSSL inactivation did not impact the gastric lipolysis level in preterm. However, *in vitro* the presence of BSSL enhanced the intestinal lipolysis disregarding the impact on gastric lipolysis. Furthermore, the potential beneficial impact of BSSL on improving fat digestion and absorption in the newborn is largely discussed in the literature and was raised in the present work. Such hypothesis justified a randomized trial that very recently studied the impact of

supplementation of HM and IF with recombinant BSSL (Casper et al., 2016). This study indicated no significant improvement in infants' growth velocity during recombinant human BSSL addition to IF or pasteurized HM. However, a favorable effect was identified for a predefined subgroup 'small for gestational age' infants. This is consistent with older studies in more vulnerable very low birth weight infants indicating that pasteurization, though the inactivation of BSSL, reduced fat absorption from human milk by approximately one third (Andersson et al., 2007; Williamson et al., 1978). Moreover, a positive effect of BSSL supplementation on velocity growth was reported in the phase 2 of the study, when only infant formula without MCFA was allowed (Casper et al., 2014). Finally, milk composition was not controlled and unbalanced lipid and energy content between groups could not be excluded. Thus, we consider that their results are not clear enough for excluding the benefits of BSSL to the infant growth and development. However, a crucial finding of this trial was the increase of the adverse events, including the risk for NEC, in infants receiving supplemented milk; alone, this finding justifies the exclusion of the supplementation with recombinant BSSL as a strategy for improving the nutritional care of infants. Thus, other possibilities should be tested concerning improved fat digestion and IF improvement.

Implementation of the ultrasonic homogenization technique in HMB: our findings presented in chapter 4 indicated that ultrasonic homogenization of PHM could be a potential strategy to improve growth and development of infants receiving banked-HM in nutritional care units. Nevertheless, the nutritional outcomes and the physiological implications of this process need to be carefully evaluated before it becomes a recommendation.

Therefore, our research group is now thinking of a new protocol for investigating this point. First of all, the digestive tolerance for receiving PHHM in all daily meals might be tested in newborns receiving exclusively banked-HM. It could be the objective of a phase 2 study, where newborns receiving PHM or PHHM would be compared, with NEC as the main criteria of evaluation and digestive troubles, such as abdominal bloating with abdominal tension, as secondary criteria. Once the tolerance is approved, a randomized controlled trial can be performed, consisting of the phase 3. The main criteria for this phase 3 would be follow the infant growth during the hospital stay. Follow-up visits during the first years of life should be planned in order to evaluate neurodevelopment.

Improving infant formulas: as demonstrated in the literature for bovine milk (*cf.* chapter 1) and confirmed in chapter 4 for HM, the HGL is very sensitive to the specific surface's increase for the same meal composition and type of milk. However, native human MFG are more susceptible to gastric lipolysis than the homogenized lipid droplets in bovine IF. Altogether, these results suggest that the quantity of surface area is not the only factor governing gastric lipolysis; the higher amount

and the type of phospholipids present in HM *versus* IF is likely to be an important factor impacting the quality of the interface, alongside with the type of homogenization applied.

Thus, these findings support the insertion of native MFG membrane in IF as crucial for mimicking HM digestive behavior. Moreover, it reinforces the necessity of testing softer technological treatments during the IF production, in order to reduce the impact on the native emulsion structure. Such hypothesis could be verified by using the *in vitro* dynamic model presented in this thesis for testing new concepts of IF.

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S.C. de Oliveira, C. Bourlieu, O. Ménard, Y. Le Gouar, A. Bellanger, E. Dirson, F. Rousseau, C. Moustiés, C. Perrier, P. Pladys, D. Dupont, A. Deglaire. Impact of pasteurization of human milk on its gastric digestion: an *in vivo* study in the preterm infant. *49th Annual Congress of ESPGHAN, Athens, Greece,* May 26-28 2016.

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C. Bourlieu & A. Deglaire, O. Ménard, S.C. de Oliveira, S. Chever, S. Bouhallab, F. Carrière, D. Dupont. Towards infant formulas mimicking human milk structure and gastric digestion? *12th EUROFED Lipid Congress, Montpellier, France*, September 14-17 2014.

A. Deglaire, S.C. de Oliveira, C. Bourlieu, O. Ménard, A. Bellanger, V. Le Bail, A. Beuchée, P. Pladys, D. Dupont. Effect of pasteurization on human milk digestion as simulated in an *in vitro* dynamic system. *19th Francophone Congress of Research in Neonatology, Paris, France*, December 16-17 2013.

Supervisions

Co-supervision of Damien Faure-Bidégaray, undergraduate student (Biologie-Environnement / Université Bretagne Sud), from April to June 2016.

Co-supervision of Célia Moustiés, master student (Master 1 Sciences et Procédés de l'Agro-alimentaire et de l'Environnement / Faculté des Sciences de Montpellier), from February to July 2015.

Poster communications

12th EUROFED Lipid Congress, Montpellier (France) 2014

Impact of pasteurisation on the digestion of human milk as simulated in an *in vitro* dynamic system

S.C. de Oliveira[1,2], C. Bourlieu[1,2], O. Ménard[1,2], A. Bellanger[3], F. Rousseau[1,2], V. Le Bail[4], P. Pladys[3], F. Carrière[5], D. Dupont[1,2], A. Deglaire[1,2]

[1]INRA UMR 1253 STLO, Rennes France; [2]Agrocampus Ouest UMR 1253 STLO, Rennes France; [3]CHU Rennes, Service de pédiatrie, France; [4]CHU Rennes, Lactarium - Unité Nutrition et Diététique Infantile, France; [5]CNRS, Aix Marseille Université, UMR7282 Enzymologie Interfaciale et de Physiologie de la Lipolyse, Marseille France

Samira.deOliveira@rennes.inra.fr

Context

Mother's milk is the optimal food for neonates (WHO)
Hospitalised newborns may be fed **pasteurised donor milk** if no mother's milk (Holder pasteurisation: 62.5°C, 30 min)

- Destruction of potential pathogens
- Alteration of immunological components (Akinbi et al., 2010; Van Gysel et al., 2012)
- Fat absorption and growth reduced in preterm infants (Andersson et al., 2007; Thomas et al., 1999)

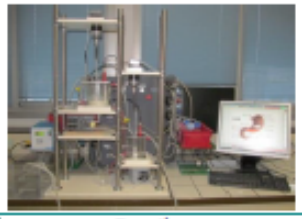
Impact of pasteurisation on digestive kinetics?

Objective

To study the impact of holder pasteurisation on the digestive kinectis of human milk.

Methods

In vitro gastro-intestinal dynamic model simulating newborn digestion (Ménard et al.; 2014; Bourlieu et al., 2014)



Pool human milk (n=5 donors) → Raw / Pasteurised → Frozen / Defrozen → Triplicate digestions

Digesta sampling - Time (min): G0 (Milk), G30 D30, G60 D60, G90 D90, G120 D120, D180

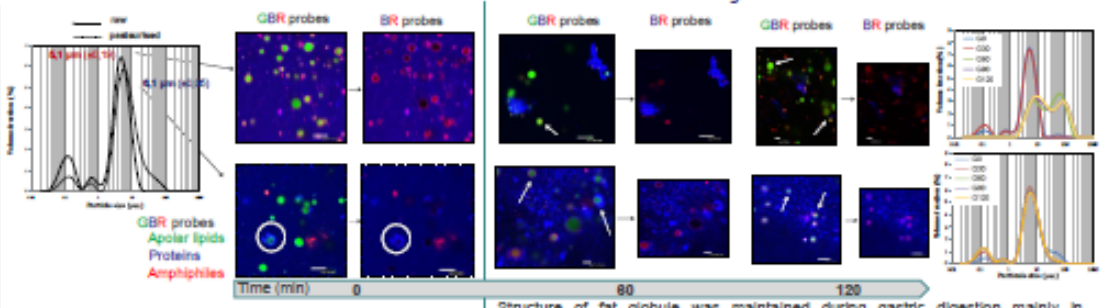
Microscopic scale — Evolution of the matrix structure: Laser light scattering, Confocal microscopy(CLSM)

Molecular scale — Lipolysis & Proteolysis: -TLC : Thin layer chromatography; - GC : Gas chromatography; -SDS-Page → Densitometry (Image QuantL) → Dilution factor correction

Results

STRUCTURE

Initial structure — Gastric digestion

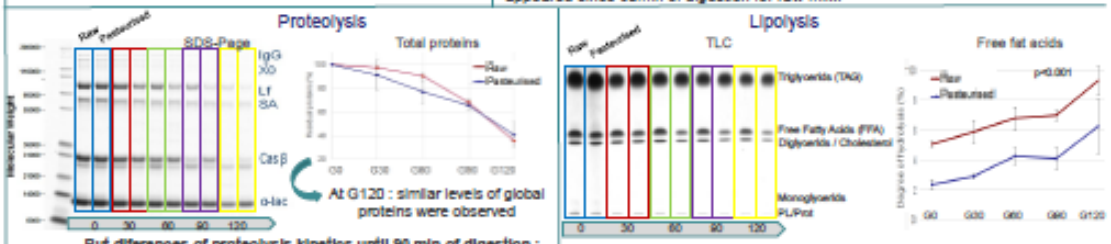


Pasteurisation leads to **aggregation of proteins** in the soluble phase and on the **interface** of the milk fat globule membrane.

Structure of fat globule was maintained during gastric digestion mainly in pasteurised milk. Mixed aggregates of apolar and amphiphilic compounds have appeared since 60min of digestion for raw milk.

HYDROLYSIS

Proteolysis — Lipolysis



At G120 : similar levels of global proteins were observed

But diferences of proteolysis kinetics until 90 min of digestion :
Casein hydrolysis faster for raw human milk ($p<0.001$)
→Aggregation after heat treatment
Lactoferrin hydrolysis faster for pasteurised milk ($p<0.05$)
→Access of proteases easier after whey proteins denaturation

Lipolysis reduced for pasteurised milk :
more TAG and less FFA at the end of the gastric phase
→Endogenous lipase in human milk inactivated by pasteurisation

Conclusion

The holder pasteurisation of human milk modifies its digestive kinectis in the gastric phase, as simulated in an *in vitro* dynamic system.

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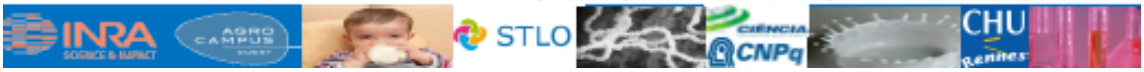
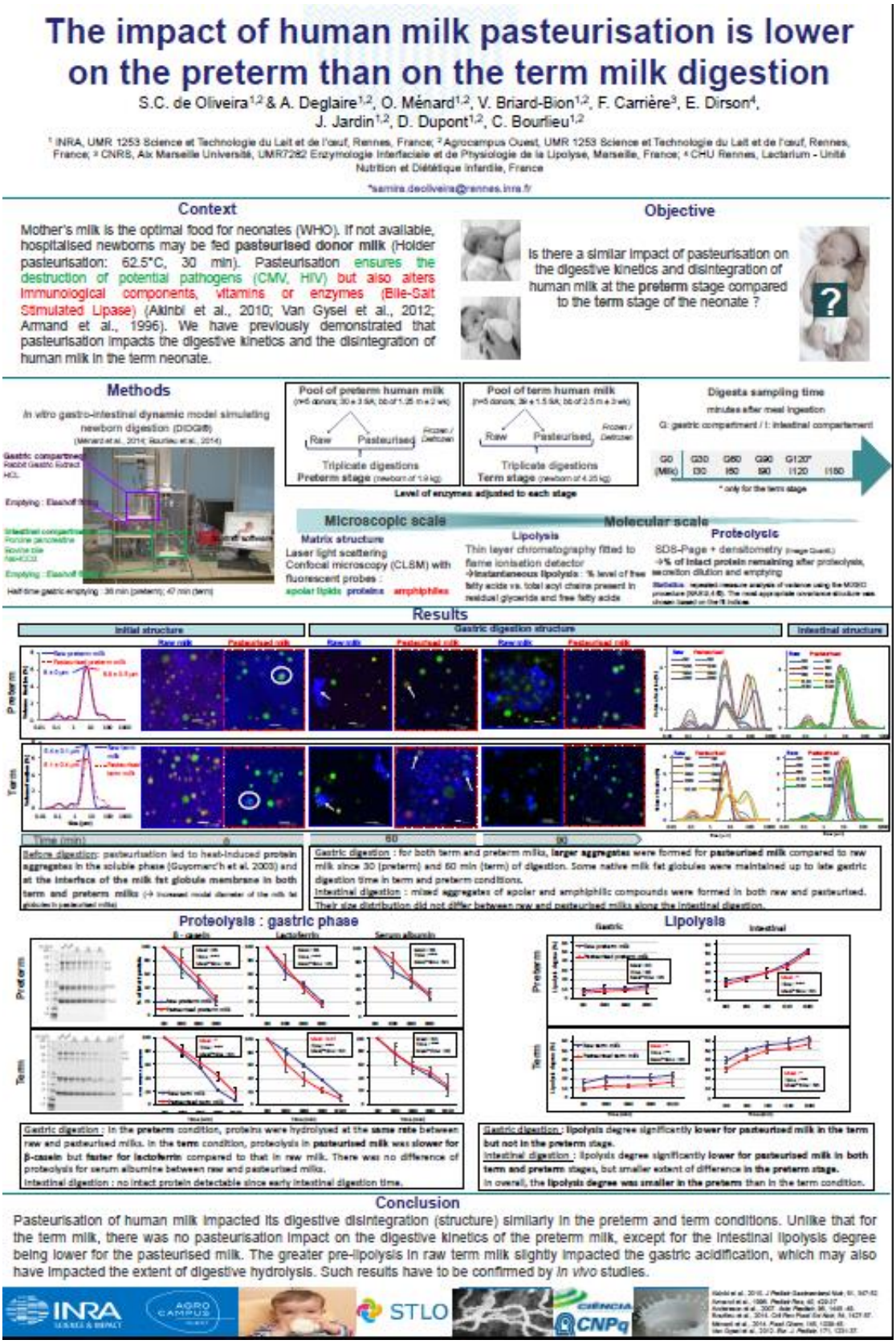


4th International Conference on Food Digestion, Naples (Italy), 2015; 3rd International Conference on Food Structures, Digestion and Health, Wellington (New Zealand), 2015 (Poster award: runner up)



3rd International Conference on Food Structures, Digestion and Health, Wellington (New Zealand), 2015

ARE INFANT FORMULAS BIOMIMETIC OF HUMAN MILK DIGESTIVE BEHAVIOR? A DYNAMIC *IN VITRO* STUDY

S. C. DE OLIVEIRA[1,2*] & C. MOUSTIES[1,2], A. DEGLAIRE[1,2], O. MÉNARD[1,2], A. BELLANGER[3], F. CARRIÈRE[4], E. DIRSON[5], Y. LE GOUAR[1,2], F. ROUSSEAU[1,2], D. DUPONT[1,2], C. BOURLIEU[1,2]

[1]INRA UMR 1253 STLO, France; [2]Agrocampus Ouest UMR 1253 STLO, France; [3]CHU Rennes, Service de pédiatrie, France; [4]CNRS, Aix Marseille Université, UMR7282 Enzymologie Interfaciale et de Physiologie de la Lipolyse, France; [5]CHU Rennes, Lactarium - Unité Nutrition et Diététique Infantile, France

*Samira.deOliveira@rennes.inra.fr, claire.bourlieu@rennes.inra.fr

Context & Objectives

Human milk is the ideal food for infant nutrition and optimal growth. When breastfeeding is not available, infant formulas are often administrated.

The chemical composition of infant formulas has been optimized but human milk structure and bioactive compounds contents are not mimicked[1]. These initial differences may modulate the kinetics of digestion and of disintegration of the emulsion in the digestive tract of the newborns.

To check this hypothesis, the digestive behaviors of a raw mature pooled human milk (RHM) and a commercial infant formula (IF) were compared using a dynamic *in vitro* digestion system

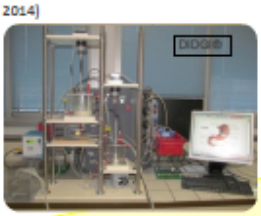
Materials & Methods

In vitro gastro-intestinal dynamic model simulating term newborn digestion (Bourlieu et al., 2014; Ménard et al., 2014)

RHM (n=2)

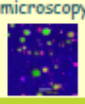
IF (n=3)

- ✓ Gradual decrease of gastric pH
- ✓ Gastric enzymes: Rabbit Gastric Extract (GERME SA)
- ✓ Intestinal enzymes: porcine pancreatine (SIGMA)
- ✓ Progressive dilution by digestive secretions
- ✓ Gastric and intestinal emptying (Elashoff fitting)



Characterization of initial matrices and their digestive behaviors

Human Milk analyzer | SDS-PAGE electrophoresis | Gas & Thin layer chromatography | Confocal scanning microscopy | Laser light scattering



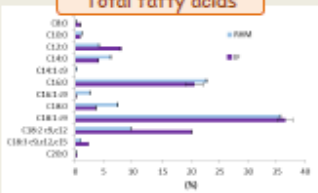
Results

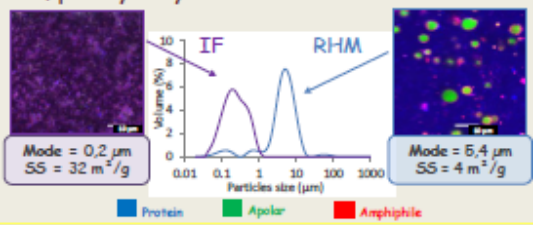
RHM and FI differs in terms of chemical composition, pre-hydrolysis state and structure

Matrices composition

g/100g	IF	RHM
Protein	1.2	1.1
Lipid	3.6	2.7
Carbohydrate	7.5	8.0

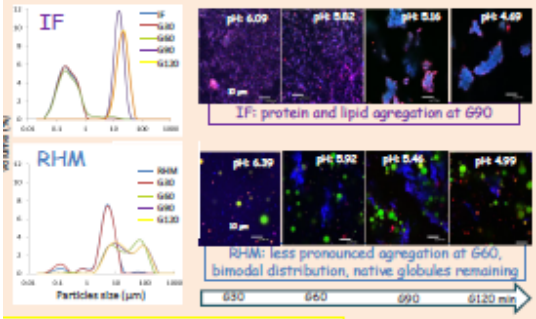
Total fatty acids

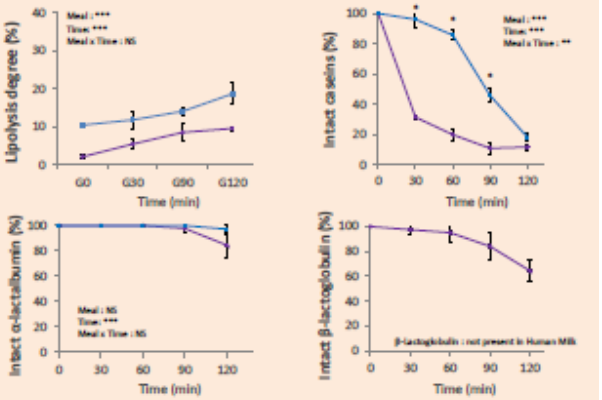




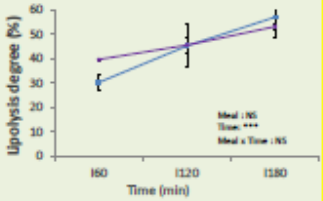
The initial differences of the two emulsions strongly impacted digestion kinetics and deconstruction

GASTRIC PHASE





INTESTINAL PHASE



→ Lipolysis level was higher in RHM than in IF before digestion and during gastric phase. This order was reversed at the beginning of the intestinal phase

→ Gastric proteolysis of caseins was accelerated in IF compared to RHM

→ Drastic proteolysis for both IF and RHM in the intestinal phase

Conclusions

Despite in constant evolution, infant formulas are not yet biomimetic of human milk digestive behavior. These differences may have important physiological implications and underline the need for developing soft processes with minimal impact on milk native emulsion structure in the near future.

*RHM: raw human milk, IF: infant formula; SS: specific surface; G: gastric phase; I: intestinal phase.

Ref: Bourlieu, C. et al. (2015). Crit Rev Food Sci Nutr, 54(11), 1427-57. Ménard, O. et al. (2014). Food Chem, 145, 1039-45.

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65, rue de Saint –Brieuc
F-35000 Rennes, FRANCE
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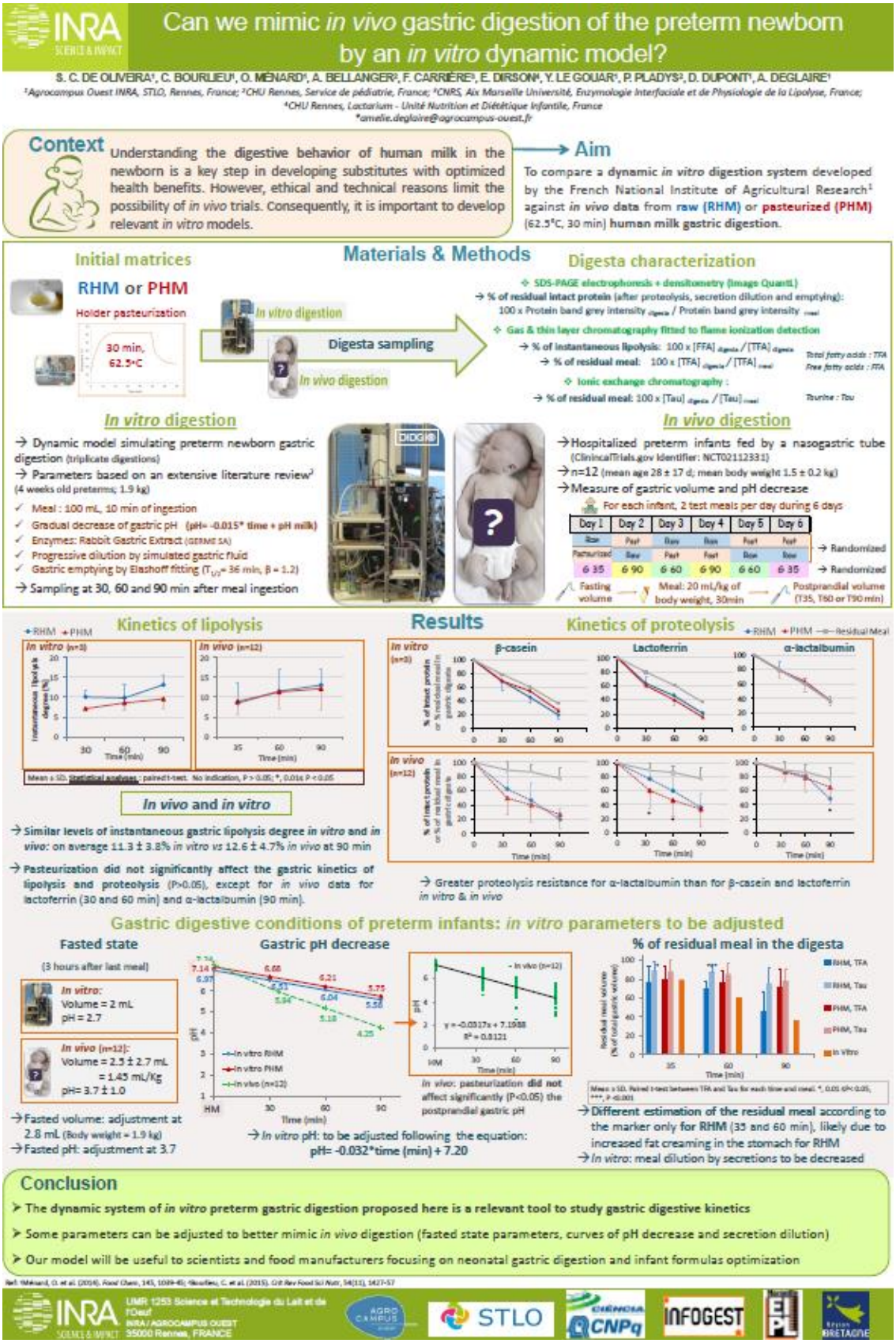
3rd International Conference on Food Structures, Digestion and Health, Wellington (New Zealand), 2015 and 16th Food Colloids Conference, Wageningen (Netherlands), 2016



49th Annual Congress of ESPGHAN, Athens (Greece), 2016

Specificity of infant gastric digestive conditions: new *in vivo* data from preterm

S. C. DE OLIVEIRA[1], A. DEGLAIRE[1], O. MÉNARD[1], A. BELLANGER[2], Y. LE GOUAR[1], E. DIRSON[1], G. HENRY[1], C. PERRIER[1], P. PLADYS[3], D. DUPONT[1], C. BOURLIEU[1]

[1]Agrocampus Ouest INRA, STLO, Rennes, France; [2]CHU Rennes, Department of Pediatrics, France; [3]CHU Rennes, Lactarium - Infant Nutrition and Dietetics, France.

*Samira.deOliveira@rennes.inra.fr; Claire.Bourlieu@rennes.inra.fr

Context & Objectives

The **digestive functions are immature during the first months of life**, especially for preterm newborns (*i.e.* motility, enzymatic and secretory functions).
The **gastric digestion** of complex colloidal emulsions such as human milk is a **key step**, which will further modulate nutrient absorption and infant nutrition. Factors such as **gastric acidification, emptying rate and enzyme outputs are key parameters** that play a role in the kinetics of hydrolysis and in the disintegration of these emulsions, and may be influenced by the nature of the meal.

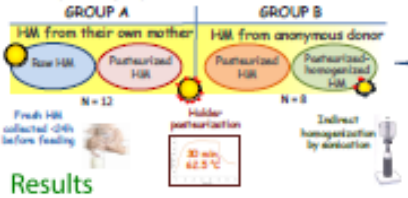
Aim: to characterize gastric contents and physiological parameters of preterm infants in fasting state or after ingestion of raw (RHM), pasteurized (PHM) or pasteurized-homogenized human milk (PHHM).

Methods

In vivo study (ClinicalTrials.gov Identifier: NCT02112331)

→ Hospitalized preterm infants fed by a nasogastric tube every 3h

→ 2 independent groups:



For each infant: **2 test meals per day during 6 days**

For each meal: **1 fasting and 1 postprandial gastric collection**

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
Meal1	Meal2	Meal2	Meal1	Meal1	Meal2	→ Randomized
Meal2	Meal1	Meal1	Meal2	Meal2	Meal1	→ Randomized
G 35	G 90	G 60	G 90	G 60	G 35	

Fasting volume — Meal: 20 mL/kg of body weight, 30min — Postprandial volume (T35, T60 or T90 min)

→ Measure of gastric volume and pH

Principle of the marker dilution

% of residual meal:

$100 \times [\text{Marker}]_{digesta} / [\text{Marker}]_{meal}$

% of secretion:

100 - % of residual meal

- Lipid marker: total fatty acids (TFA)
  Gas chromatography fitted to flame ionization detection
- Soluble marker: free amino acid taurine (Tau)
  Ionic exchange chromatography

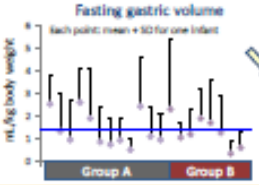
Results

Subjects

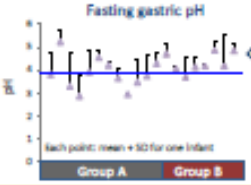
Characteristics	Group A (n = 12)	Group B (n = 8)
Gestational age	30 ± 1.1 wk	29.5 ± 1.5 wk
Age at D1 of the study	27 ± 12 d	32 ± 21 d
Body weight at D1 of the study	1.8 ± 0.4 kg	1.8 ± 0.5 kg

Mean ± SD

Fasting state (3h after last meal)



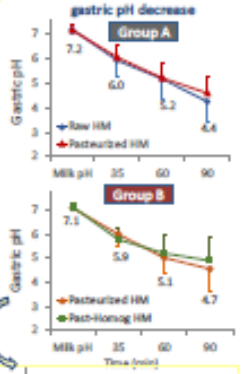
Overall mean ± SD
(n = 225 points)
1.40 ± 1.64 mL/kg
(min – max : 0 – 9.72 mL/kg)



Overall mean ± SD
(n = 86 points)
3.94 ± 0.90
(min – max : 1.68 – 5.59)

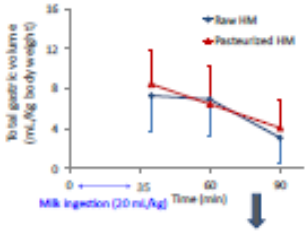
Postprandial state

Technological treatments did not impact gastric pH decrease

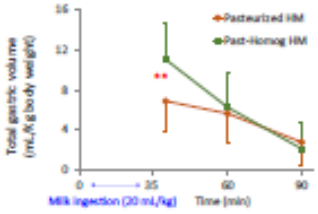


→ **Capacity of acidification not as weak as expected**

Group A: Pasteurization did not impact total gastric volume



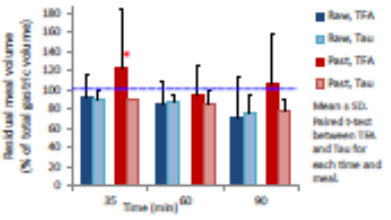
Group B: Homogenization impacted total gastric volume at 35 min



Lipolyse ++ for PHHM: Hormonal feedback delay gastric emptying

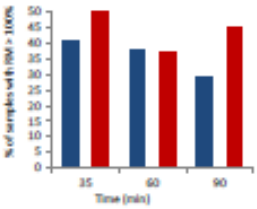
The estimation of residual meal on the total gastric volume differed depending on the marker

Mean % of residual meal in the total gastric volume



- **Different estimation of the residual meal** according to the marker only for PHM at 35 min.

Proportion of samples with a residual meal value > 100% - Lipid marker



- **Creaming suggested by % of residual meal > 100%**

Change of state of dispersion

→ **Non-homogeneous emptying of both raw and pasteurized HM: emptying faster for the liquid phase of the emulsion**

(Guldig et al., 2011; Guldig & Wooster, 2013; Mandavi et al., 2009)

Statistical significance: **, $p < 0.01$; *, $p < 0.05$. When no sign is reported, p-value > 0.05.

Conclusion

This study presents a **unique set of data illustrating the specificity of preterm infant gastric digestive conditions.** Understanding the physiological environment in the infant gastric tract is essential to **optimize neonatal nutrition** and oral drug delivery. Moreover, these data will be useful to develop relevant *in vitro* models of infant digestion.

Disclosure of interest: None declared
RHM: raw human milk; PHM: pasteurized human milk; PHHM: pasteurized and homogenized human milk.
Acknowledgements: COST action FA1005 INFOGEST; the volunteer mothers and the colleagues from the bank milk of the Rennes University Hospital Center; CNPq Brazil.

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65, rue de Saint-Brieuc
F-35000 Rennes, FRANCE
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1-Guldig, M., Wooster, T.J., Day, L., et al. (2011). Impact of gastric structuring on the hydrolysis of emulsified lipids. pp. 1510-1520. 2-Guldig, M. & Wooster, T.J. (2013). The influence of emulsion structure and stability on lipid digestion. Current Opinion in Colloid & Interface Science 18, 90-101. 3- Mandavi, L., Whitham, M., Wright, J., et al. (2009). Magnetic resonance imaging insights into how fat emulsion stability affects gastric emptying. Gastroenterology 136, A581.

49th Annual Congress of ESPGHAN, Athens (Greece), 2016 (E-poster with flash presentation)

Gastric lipase and other lipolytic enzymes activity in the preterm fed raw, pasteurized or pasteurized-homogenized human milk

S.C. De Oliveira[1], Y. Le Gouar[1], O. Ménard[1], D. Faure-Bidégaray[1], A. Bellanger[2], E. Dirson[3], P. Pladys[2], F. Carrière[4], D. Dupont[1], A. Deglaire[1], C. Bourlieu[1]

[1]Agrocampus Ouest INRA, STLO, Rennes, France; [2]CHU Rennes, Department of Pediatrics, France; [3]CHU Rennes, Lactarium - Infant Nutrition and Dietetics, France; [4]CNRS-Aix-Marseille University-UMR 7282 EIPL, Marseille, France.

*Samira.deOliveira@rennes.inra.fr, Claire.Bourlieu@rennes.inra.fr

Context

Human gastric lipase (HGL):

- initiates the digestion of milk lipids and compensates for the immaturity of exocrine pancreatic function in preterm newborns
- favors the subsequent action of other enzymes such as pancreatic or bile salt stimulated lipases
- activity in gastric aspirates of infants in fasting or postprandial state: high variability probably linked to meal nature, infant age or analysis method

Human pancreatic lipase (HPL):

- Present in gastric aspirates only if duodenal reflux

Bile salt-stimulated lipase (BSSL):

- Naturally present in human milk but inactivated by Holder pasteurization

Objectives

To determine **HGL activity, output and the contribution of other lipolytic enzymes** in gastric aspirates of preterm infants in **fasting state or after administration** of raw (RHM), pasteurized (PHM) or pasteurized-homogenized human milk (PHHM).

Methods

In vivo study (ClinincalTrials.gov Identifier: NCT02112331)

→ Hospitalized preterm infants fed by a nasogastric tube

→ 2 independents groups:

GROUP A	GROUP B
HM from their own mother	HM from anonymous donor
Raw HM, Pasteurized HM	Pasteurized HM, Pasteurized-homogenized HM
N = 12, ID = 1 to 12	N = 8, ID = 13 to 20

For each infant, **2 test meals per day during 6 days**

For each meal, **1 fasting and 1 postprandial gastric collection**

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
Meal1	Meal2	Meal2	Meal1	Meal1	Meal2	→ Randomized
Meal2	Meal1	Meal1	Meal2	Meal2	Meal1	→ Randomized
G 35	G 90	G 60	G 90	G 60	G 35	

Fasting volume → Meal: 20 mL/kg of body weight, 30min → Postprandial volume (T35, T60 or T90 min)

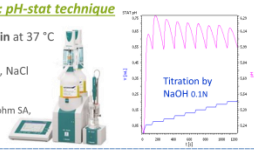
→ Samples were blended with glycerol (50:50 v/v) and frozen (-20 °C) after addition of antiprotease

Lipolytic enzymes activity measurements: pH-stat technique

→ Titration of butyric acid released from **tributyrin** at 37 °C (Gargouri et al., 1986)

→ Assay solution: sodium taurodeoxycholate (2mM), NaCl (150 mM), bovine serum albumin (0.1 g/L), H_2O

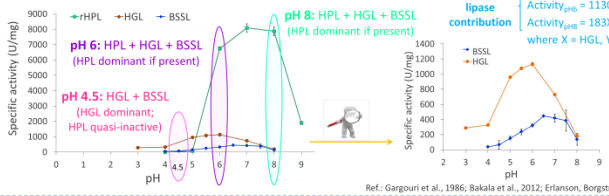
pH-stat device: Titrando 842, Metrohm SA, Courtaboeuf, France



-Activity expressed as enzyme units per mL of gastric content (U/mL).

-1 U = release of 1 µmol of butyric acid per minute under the assay conditions, and at a given pH

Determination of lipolytic enzymes activity at three different pH:



Individual lipase contribution

$Activity_{pH4.5} = 643.8X+65Y$

$Activity_{pH6} = 1130X+320Y+6743Z$

$Activity_{pH8} = 183X+135Y+7872Z$

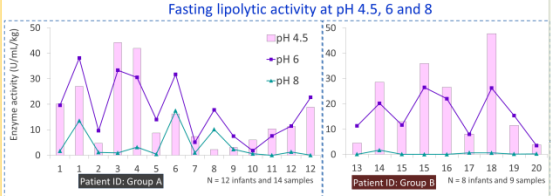
where X = HGL, Y = BSSL, Z = HPL

Ref.: Gargouri et al., 1986; Bakala et al., 2012; Erlanson, Borgström & Scand , 1970

Results

FASTING STATE and individual lipase contribution

Fasting lipolytic activity at pH 4.5, 6 and 8



✓ pH 4.5: activity = 15.9 ± 3.8 U/mL/kg and 141.6 ± 26.0 total U/kg

✓ pH 8: mean contribution of HPL = 2.3 ± 0.7 µg/mL global activity > 5 U/mL/kg for 3 infants:

✓ pH 4.5: activity = 19.9 ± 5.1 U/mL/kg and 78.7 ± 28.6 total U/kg

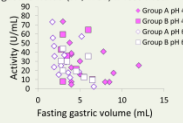
✓ pH 8: no aliquot with active BSSL global activity < 5 U/mL/kg

HGL output: specific activity *vs* pH curves

System of equations which determines the theoretical contribution of each lipase in the aspirate

→ No difference between groups: HGL = 61 ± 14 µg/mL (group A) and 69 ± 19 µg/mL (group B) - Mean ± SEM (p=0.5, T-test)

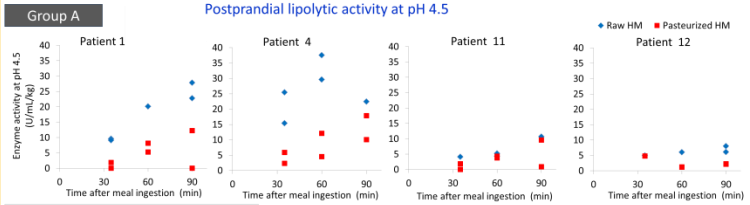
✓ **Lipolytic activity** (pH 4.5 and 6): **inversely correlated with fasting gastric volume and pH** => more concentrated gastric fluids (H^+, HGL) if less meal



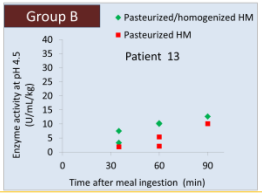
POSTPRANDIAL KINETICS

Postprandial lipolytic activity at pH 4.5

Group A



Group B



✓ BSSL from RHM contributing to lipolytic activity at pH 4.5 but HGL dominant: increase of activity with time and meal emptying

✓ Meal induced secretion of HGL: higher for RHM but high inter-individual variability

✓ Group B meal induced secretion of HGL: higher for P+HHM but only 1 patient (ongoing analyses)

Refs: Bakala N'Goma et al., 2012; Borel et al., 1991; Lengsfeld et al. 2004; Moreau et al., 1988a; Moreau et al., 1988b

Conclusions

This study presents a unique set of data illustrating the specificity of preterm infant gastric digestive conditions. These data will be useful to develop relevant *in vitro* models of infant digestion and analyze the link between gastric lipolytic activity and lipid digestion.

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Disclosure of interest: None declared

BSSL: bile salt stimulated lipase; HGL: human gastric lipase; HPL: human pancreatic lipase; RHM: raw human milk; PHM: pasteurized human milk; P+HHM: pasteurized and homogenized human milk.

Acknowledgements: COST action FA1005 INFOGEST; the volunteer mothers and the colleagues from the bank milk of the Rennes University Hospital Center; CNPq Brazil.

ESPGHAN 49th ANNUAL MEETING

Abstract

Impact of pasteurization and homogenization on the digestion of human milk in the newborn infant: *in vitro* and *in vivo* studies

Human milk (HM) is the gold standard in neonatal nutrition of term and preterm infants. When breastfeeding is not possible, pasteurized HM (PHM) from milk banks is preferentially administered instead of infant formula, especially for vulnerable hospitalized newborns. Holder pasteurization (62.5°C, 30 min), applied for safety reasons, may reduce fat absorption through inactivation of milk endogenous lipases. This could be counteracted by homogenization of PHM through an increase of the specific surface available for enzyme adsorption. In this context, the main objective of this thesis was to study the impact of Holder pasteurization and ultrasonic homogenization on the digestion of HM in the newborn, and more specifically on the kinetics of lipolysis, proteolysis and structural disintegration. To this aim, an *in vitro* dynamic model was used to evaluate the gastrointestinal digestion of raw HM (RHM) *vs.* PHM at preterm and term stages. Then, an *in vivo* study was conducted on hospitalized preterm newborns for comparing the gastric digestion of (A) RHM *vs.* PHM and (B) PHM *vs.* pasteurized-homogenized HM (PHHM). *In vivo,* gastric conditions in fasting and postprandial states were characterized in terms of pH, total volume, emptying rate and lipolytic activities.

We demonstrated that pasteurization and homogenization affected the HM initial structure and its digestive kinetics and structural disintegration. While gastric lipolysis was reduced after pasteurization in term *in vitro* study (24.1 ± 3.2% *vs.* 16.5 ± 5% at 120 min for RHM and PHM, respectively), no impact was observed on the gastric lipolysis degree at the preterm stage. Intestinal lipolysis, evaluated *in vitro* only, was reduced by pasteurization at preterm and term stages. Gastric protein digestion was selectively affected by pasteurization; *in vivo,* pasteurization enhanced proteolysis of lactoferrin and reduced that of α-lactalbumin (only at 90 min). *In vivo*, homogenization increased the lipolysis degree, the difference being greater at 35 and 60 min (respectively 22 and 24% higher for PHHM). In addition, homogenization enhanced the proteolysis of serum albumin. Physiological gastric conditions during digestion were little affected by technological treatments: administration of RHM enhanced postprandial lipolytic activity (at 90 min, 60% higher for RHM *vs.* PHM), homogenization slowed down gastric emptying (half emptying time estimated at 30 *vs.* 38 min for PHM and PHHM, respectively) and pH decrease was not affected by the type of milk.

Overall, the *in vivo* data described here are crucial for a better understanding of infant digestive conditions and may help to improve the setup of *in vitro* digestion models. Our findings may be useful to support the challenging nutritional management of preterm infants.

Résumé

Impact de la pasteurisation et de l'homogénéisation sur la digestion du lait maternel chez le nouveau-né: études *in vitro* et *in vivo*

Le lait maternel (LM) est l'aliment de référence pour les nourrissons nés à terme ou prématurés. Lorsque l'allaitement est impossible, le LM pasteurisé (LMP) provenant de banque de lait (lactarium) est préférentiellement administré par rapport aux formules infantiles, en particulier pour les nouveau-nés hospitalisés vulnérables. La pasteurisation de Holder (62,5°C, 30 min), appliquée pour des raisons sanitaires, peut réduire l'absorption des lipides par l'inactivation des lipases endogènes du LM. L'homogénéisation du LMP pourrait contrer cet effet adverse en augmentant la surface spécifique disponible. Dans ce contexte, l'objectif principal de cette thèse était d'étudier l'impact de la pasteurisation de Holder et de l'homogénéisation par ultrasons sur la digestion du LM chez le nouveau-né. Pour cela, un modèle de digestion *in vitro* était utilisé pour évaluer la digestion gastro-intestinale de LM cru (LMC) *vs.* LMP chez des nouveau-nés à terme et prématurés. Ensuite, une étude *in vivo* a été menée chez des nouveau-nés prématurés hospitalisés pour comparer la digestion gastrique de (A) LMC *vs.* LMP et (B) LMP *vs.* LM pasteurisé et homogénéisé (LMPH). *In vivo*, les conditions gastriques à jeun et postprandiales ont été caractérisées en termes de pH, volume total, taux de vidange et activités lipolytiques.

Nous avons démontré que la pasteurisation et l'homogénéisation ont affecté la structure initiale du LM, ses cinétiques digestives et sa désintégration structurelle. Alors que la lipolyse gastrique a été réduite après la pasteurisation dans l'étude *in vitro* à terme (24,1 ± 3,2% *vs*. 16,5 ± 5% à 120 min pour LMC et LMP, respectivement), aucun impact n'a été observé sur le degré de lipolyse gastrique au stade prématuré. La lipolyse intestinale, évaluée seulement *in vitro*, a été significativement réduite aux deux stades, prématuré et à terme, à des niveaux différents. La digestion gastrique des protéines a été sélectivement affectée par la pasteurisation ; *in vivo*, la pasteurisation a accéléré la protéolyse de la lactoferrine et a réduit celle de l'α-lactalbumine (seulement à 90 min). *In vivo*, l'homogénéisation a accéléré la lipolyse, l'écart étant supérieur à 35 et à 60 min (respectivement 22 et 24% plus élevé pour LMPH). De plus, l'homogénéisation a augmenté la protéolyse de l'albumine sérique. Les conditions physiologiques lors de la digestion gastrique ont été peu affectées par les traitements : l'administration de LMC a augmenté l'activité lipolytique postprandiale (à 90 min, 60% plus élevée pour LMC *vs.* LMP), l'homogénéisation a ralenti la vidange gastrique (temps de demi-vidange estimé à 30 et 38 min pour LMP et pour LMPH, respectivement) et le pH postprandial n'a pas été affecté par le type de lait.

Dans l'ensemble, les données *in vivo* décrites ici sont cruciales pour une meilleure compréhension des conditions digestives chez les nourrissons et peuvent contribuer à améliorer la mise en place de modèles de digestion *in vitro*. Nos résultats peuvent être utiles pour soutenir la prise en charge nutritionnelle difficile des nouveau-nés prématurés.

RÉSUMÉ

Impact de la pasteurisation et de l'homogénéisation sur la digestion du lait maternel chez le nouveau-né : études *in vitro* et *in vivo*

Lorsque l'allaitement est impossible, du lait maternel pasteurisé (LMP) est préférentiellement administré, en particulier aux nouveaux-nés hospitalisés. La pasteurisation de Holder (62,5 °C; 30 min) est appliquée pour des raisons sanitaires mais pourrait réduire l'absorption des lipides via l'inactivation des lipases endogènes du lait. L'homogénéisation du LMP pourrait contrer cet effet négatif en augmentant la surface disponible pour l'adsorption des enzymes. L'objectif de cette thèse était d'étudier l'impact de la pasteurisation de Holder et de l'homogénéisation par ultrasons sur la digestion du LM chez le nouveau-né. Un modèle de digestion in vitro a été mis en place pour évaluer la digestion gastro-intestinale de LM cru (LMC) vs. LMP aux stades « nouveau-né à terme » ou « prématuré ». Une étude clinique a été menée chez des nouveau-nés prématurés pour comparer la digestion gastrique de (A) LMC vs. LMP et (B) LMP vs. LM pasteurisé et homogénéisé (LMPH). La pasteurisation et l'homogénéisation ont modifié la structure initiale du LM, ses cinétiques digestives et sa désintégration structurale. In vitro, la pasteurisation a réduit la lipolyse gastrique au stade à terme, alors qu'aucun impact n'a été observé au stade prématuré. La lipolyse intestinale, in vitro, a été réduite. In vivo, la pasteurisation a accélérée la protéolyse gastrique de la lactoferrine et a réduit celle de l'α-lactalbumine. L'homogénéisation a accéléré la lipolyse et la protéolyse de l'albumine sérique. Concernant les conditions physiologiques, l'activité lipolytique postprandiale était augmentée après administration de LMC et la vidange était ralentie après administration de LMPH. Les données in vivo décrites ici peuvent contribuer à améliorer les modèles de digestion in vitro mais aussi à soutenir la prise en charge nutritionnelle difficile des nouveau-nés prématurés.

Mots-clefs : digestion, pasteurisation de Holder, homogénéisation, lait maternel, lipolyse, protéolyse.

ABSTRACT

Impact of pasteurization and homogenization on the digestion of human milk in the newborn infant:* in vitro *and* in vivo *studies

When breastfeeding is not possible, pasteurized human milk (PHM) from milk banks is preferentially administered, especially for vulnerable hospitalized newborns. Holder pasteurization (62.5 °C, 30 min) is applied for sanitary reasons but may reduce fat absorption through inactivation of milk endogenous lipases. This could be counteracted by homogenization of PHM through an increase of the specific surface available for enzyme adsorption. The objective of this thesis was to study the impact of Holder pasteurization and ultrasonic homogenization on the digestion of HM in the newborn. An in vitro dynamic model was used to evaluate the gastrointestinal digestion of raw HM (RHM) vs. PHM at preterm and term stages. A clinical trial was conducted on hospitalized preterm newborns for comparing the gastric digestion of (A) RHM vs. PHM and (B) PHM vs. pasteurized-homogenized HM (PHHM). Pasteurization and homogenization affected the HM initial structure and its digestive kinetics and structural disintegration. While gastric lipolysis was reduced after pasteurization in term in vitro study, no impact was observed at the preterm stage. Intestinal lipolysis, in vitro, was reduced by pasteurization. Gastric proteolysis was selectively affected by pasteurization, being, in vivo, faster for lactoferrin and slower for α-lactalbumin. Homogenization increased lipolysis and proteolysis of serum albumin. Some physiological gastric conditions were affected by treatments: RHM had enhanced postprandial lipolytic activity and PHHM had a reduced gastric emptying time. The in vivo data described here may help to improve the setup of in vitro digestion models, but also to support the challenging nutritional management of preterm infants.

Keywords: *digestion, Holder pasteurization, homogenization, human milk, lipolysis, proteolysis.*

AGROCAMPUS OUEST • Institut supérieur des sciences agronomiques, agroalimentaires, horticoles et du paysage
65 rue de Saint-Brieuc – CS84215 – F-35042 Rennes Cedex
Tél. : 02 23 48 50 00
www.agrocampus-ouest.fr