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

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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.

1. Introduction

Digestion of nutrients in newborns is essential for their optimized growth and development, but some of their digestive functions are immature at birth (Hamosh, 1983; Lindquist & Hernell, 2010). 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 World Health Organization (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., 2015; Hamosh, 1983; Lonnerdal, 2014). 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, Underwood, Zivkovic, & German, 2012; Goldman, 2000). Therefore, human milk feeding is an important recommendation on intervention strategies to improve preterm birth outcomes (World Health Organization, 2015).

Because of improved outcomes compared to infant formulas, *i.e.* on tolerance feeding, preventing inflammation and infections (Quigley & McGuire, 2014; Schanler, Lau, Hurst, & Smith, 2005), pasteurized human milk from milk banks is the second choice after fresh milk from the own mother (Arslanoglu et al., 2013; Picaud, 2015). However, as extensively reported, processing (collection, freeze-thaw cycles) and pasteurization of human milk modify its nutritional and biological quality (O'Connor, Ewaschuk, & Unger,

Abbreviations: AA, amino acid; BSSL, bile-salt stimulated lipase; CLSM, confocal laser scanning microscopy; FA, fatty acid; PHM, pasteurized human milk; RHM, raw human milk; SS, specific surface area.

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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 (de Oliveira et al., 2015; Deglaire et al., 2016). 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. Materials and methods

2.1. Materials

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

2.2. 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 26–34 years. Milk samples were expressed on average 6.6 ± 2.4 weeks after preterm delivery (range: 4–10 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).

2.3. 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 1), 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, and 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.

Table 1
Gastrointestinal parameters for *in vitro* dynamic digestion of human milk simulating preterm conditions.

Gastric conditions (37 °C)		
SGF (stock solution adjusted at pH 6.5)	Na^+	118 mmol/L
	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 (Elashoff fitting)	$t_{½}$ β	36 min 1.15
Intestinal conditions (37 °C)		
SIF (stock solution adjusted at pH 6.2)	Na^+	140 mmol/L
	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 (Elashoff fitting)	$t_{½}$ β	200 min 2.2

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

Digestion experiments were performed in triplicate for each matrix, over 3 h. 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.

2.4. 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