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EFEITO DA PASTEURIZAÇÃO E DA ALTA PRESSÃO EM LEITE E COLOSTRO HUMANO

EFFECT OF PASTEURISATION AND HIGH PRESSURE IN HUMAN MILK AND COLOSTRUM

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia - Ramo Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Ivonne Delgadillo Giraldo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro.

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Non ducor, duco

palavras-chave

Leite humano, colostro, pasteurização, alta pressão, imunoglobulinas, lisozima, lactoperoxidase, fatores bioativos, bancos de leite.

resumo

A amamentação é o modo ideal de nutrir os bebés. Estimula o sistema imunitário, a digestão e absorção dos nutrientes, a função gastrointestinal e o desenvolvimento neurológico do bebé. A composição do leite materno varia ao longo do ciclo de lactação, sendo que o colostro – primeira secreção da glândula mamária – é mais rico em nutrientes e anticorpos. O leite materno apresenta benefícios imunológicos e nutricionais acrescidos relativamente às fórmulas infantis à base de leite de vaca. Assim, quando uma mãe não pode amamentar, o leite de outras mães (dadoras) é a alternativa mais adequada, existindo bancos de leite humano em vários países para este fim. Nestes bancos, o leite é comummente pasteurizado pelo método Holder (62.5 ºC durante 30 min), de modo a garantir a sua segurança microbiológica. No entanto, esta pasteurização lenta resulta em diversas perdas nutricionais e funcionais. Como tal, este leite pasteurizado tem de ser suplementado com multi-nutrientes. A alta pressão (AP) é uma tecnologia cada vez mais usada na indústria alimentar, uma vez que permite pasteurizar os alimentos à temperatura ambiente ou mesmo a frio, garantindo a sua segurança microbiológica com manutenção das propriedades nutricionais e funcionais.

Este trabalho teve como objetivo primordial avaliar e comparar o efeito da pasteurização Holder e da AP (200, 400 e 600 MPa durante 2.5, 15 e 30 min a 8 ºC) na concentração das imunoglobulinas A, M e G, e na atividade das enzimas lisozima e lactoperoxidase do leite e colostro humanos. Observou-se que tratamentos de AP a 200 e 400 MPa não causaram diminuções significativas em todas as Igs e na lisozima ($p>0.05$). A pasteurização, por sua vez, resultou em perdas significativas destas proteínas antimicrobianas. Não foi detetada atividade da enzima lactoperoxidase. No caso do leite humano, também o efeito combinado da AP com alta temperatura (500, 700 e 900 MPa a 40, 60 e 80 ºC durante 15 min) na atividade da lisozima foi testado, tendo-se verificado um aumento na atividade após 500, 700 e 900 MPa a 40 ºC e a inativação da enzima a 80 ºC. Além disto, é apresentada uma revisão detalhada da literatura acerca da composição do leite humano, bancos de leite e efeitos da pasteurização Holder, tecnologia de AP e efeito da mesma no referido leite.

keywords

Human milk, colostrum, pasteurisation, high pressure, immunoglobulins, lysozyme, lactoperoxidase, bioactive factors, milk banks.

abstract

Breastfeeding is the ideal nutrition for infants. It enhances host defences, digestion and absorption of nutrients, gastrointestinal function, and neurological development. Breast milk composition varies widely along lactation, and colostrum – first secretion of the mammary gland – is richer in nutrients and immunoglobulins (Igs). Human milk is a better alternative than bovine milk-based infant formulas, due to its unique immunological and nutritional profile. Therefore, when the mother cannot breastfeed, the breast milk of other mothers (donors) is the most adequate alternative. There are human milk banks in several countries for this purpose, which commonly pasteurise the milk by the Holder pasteurisation method (62.5 ºC for 30 min), in order to assure its microbial safety. However, this low-temperature long-time pasteurisation results in various nutritional and functional losses. Thus, this pasteurised milk has to be supplemented with multi-nutrients. High-pressure processing (HPP) is a processing technology that is being increasingly applied in the food industry, since it allows pasteurisation of foods at room temperature or even at refrigeration temperature, guarantying microbial inactivation while maintaining its nutritional and functional properties.

The main goal of this work was to investigate and compare the effect of Holder pasteurisation and HPP (200, 400 and 600 MPa for 2.5, 15 and 30 min at 8 ºC) in IgA, IgM and IgG concentrations, and lysozyme and lactoperoxidase activity in human milk and colostrum. We found that HPP treatments at 200 and 400 MPa did not cause significant decreases in all of the Igs and lysozyme ($p>0.05$). Holder pasteurisation, on the other hand, led to significant losses of these antimicrobial proteins. Lactoperoxidase activity was not detected. The combined effect of HPP and high temperature (500, 700 and 900 MPa at 40, 60 and 80 ºC for 15 min) in lysozyme activity of human milk was also studied. We observed an increase in lysozyme activity after 500, 700 and 900 MPa at 40 ºC, and inactivation of the enzyme at 80 ºC. Moreover, a detailed literature review about human milk composition, milk banks and Holder pasteurisation effects, and HPP technology and its effects on human milk is presented.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid
ALA	α-linolenic acid
BSSL	Bile salt-stimulated lipase
Ca	Calcium
Cl	Chlorine
CLA	Conjugated linoleic acid
CMV	Cytomegalovirus
Cu	Cooper
D	Decimal reduction time
DHA	Docosahexaenoic acid
ESPGHAN	European Society of Paediatric Gastroenterology, Hepatology and Nutrition
Fe	Iron
FFAs	Free fatty acids
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HMB	Human milk banks
HMBANA	Human milk banking association of North America
HPP	High-pressure processing
HTLV	Human T cell lymphotropic virus
I	Iodine
IFN-γ	Interferon-γ
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Igs	Immunoglobulins
IL-1β	Interleukin-1β
IL-10	Interleukin-10
k	Reaction rate constant
K	Potassium
LA	Linoleic acid
LPL	Lipoprotein lipase

LPO	Lactoperoxidase
LTLT	Low-temperature long-time
Mg	Magnesium
Na	Sodium
NEC	Necrotising enterocolitis
NICU	Neonatal intensive care unit
NPN	Non-protein nitrogen
P	Phosphorous
PEF	Pulsed electric field
PME	Pectin methyl esterase
PUFAs	Poly-unsaturated fatty acids
Se	Selenium
sIgA	Secretory immunoglobulin A
sIgM	Secretory immunoglobulin M
UNICEF	The United Nations Children's Fund
USDA	The United States Department of Agriculture
UV-Vis	Ultraviolet-visible
TGF-α	Transforming growth factor-α
TGF-β2	Transforming growth factor-β2
TNF-α	Tumor necrosis factor-α
WHO	The World Health Organization
Zn	Zinc

Contextualisation and Thesis Structure

The present work is divided into four different chapters. Chapter 1 consists of a comprehensive literature review and state of the art in what concerns human milk composition, human milk banks, the currently used thermal pasteurisation method and the non-thermal high-pressure processing (HPP) technology. Chapters 2 and 3 consist of a comparative evaluation of thermal pasteurisation and HPP effects on immunoglobulin content and antimicrobial enzyme activity in human colostrum and human milk, respectively. Each one of these last two chapters contains an individual introduction, material and methods, and results and discussion section. Moreover, the three chapters above referred have individual abstract, conclusions and references sections. Chapter 4 features the global conclusions of the previous chapters and an analysis of the future work to be done in this research area. The criterion in the basis of this division is the fact that the first three chapters will constitute three distinct scientific papers, to be published in international peer-reviewed scientific journals.

CHAPTER 1

Human Milk: Evaluating High-Pressure Processing as a Non-Thermal Pasteurisation Technology

Abstract

Human milk is currently seen not only as a food, but as a dynamic biologic system. It provides nutrients, bioactive components and immune factors, promoting adequate and healthy growth of newborn infants. When mothers cannot supply their children, donated breast milk is the nutrition recommended by the World Health Organization (WHO), as it is a better alternative than infant formula. However, because of the manner in which donor milk is handled in human milk banks (HMB) many of the properties ascribed to mother's own milk are diminished or destroyed. The major process responsible for these losses is Holder pasteurisation. High-pressure processing (HPP) is a novel non-thermal pasteurisation technology that is being applied in food industries worldwide primarily as an alternative to thermal treatment, due to its capacity of inactivating microorganisms while preserving both nutritional and bioactive components of foods. This review describes human milk composition and critically discusses HMB importance and practices, highlighting HPP as a potential pasteurisation technology for the human milk. HPP technology is described and the currently existing studies of its effects in human milk presented.

1. Introduction

During the last decades multiple scientific data have confirmed that breastfeeding is the optimum nutrition for term and preterm infants, at least for the first six months and, if possible, for the first year of life or even more (Bertino *et al.*, 2009). The World Health Organization (WHO) and The United Nations Children's Fund (UNICEF) recommend exclusive breastfeeding for the first six months of life and the introduction of complementary foods at six months together with continued breastfeeding up to two years and beyond (World Health Organization, 2010). Human milk provides several benefits to the infants, including enhancement of host defences, neurological development and gastrointestinal function (Heiman & Schanler, 2007; Lawrence & Pane, 2007). In addition, it enhances antioxidant defences against hydroxyl radical aggression in preterm infants (Ledo *et al.*, 2009).

Thus, besides the role that breastfeeding plays in the normal development of the infant, it also protects against infectious diseases in infancy and childhood. The protective effect of breast milk against diarrhea, respiratory and urinary tract infections, necrotising enterocolitis (NEC), nosocomial sepsis, otitis media and common infections in premature infants has been documented (El-Mohandes *et al.*, 1997; Lawrence & Pane, 2007; Marild *et al.*, 2004; McGuire & Anthony, 2003). Early prospective studies reported lower rates of infection in premature infants being fed fresh human milk when compared with formula milk (Narayanan *et al.*, 1980; Narayanan *et al.*, 1984). In the case of NEC, a published meta-analysis revealed that infants receiving human milk were three times less likely to develop NEC than infants receiving formula milk (McGuire & Anthony, 2003). Another systematic review and meta-analysis suggests that human milk might reduce the risk of NEC by about 79% when compared to formula milk (Boyd *et al.*, 2007). Breastfeeding has also proven to have a protective effect against obesity in children (Arenz *et al.*, 2004).

2. Human milk composition

Human milk is currently seen not only as a food, but also as a dynamic biologic system. This complex fluid simultaneously provides nutrients, bioactive components and immune factors like immunoglobulins (Igs), lactoferrin and lysozyme (Field, 2005; Lopez Alvarez, 2007). The composition of human breast milk is not homogeneous; it varies over the course of lactation and between lactating women. It is known that many factors, such as weeks of lactation, breastfeeding time, gestational age, genetic factors and dietary habits are responsible for the variations in human milk components between individuals (Maas *et al.*, 1998; Wojcik *et al.*, 2009). In a single individual, breast milk composition may even change over the course of the day (Shubat *et al.*, 1989) and significantly from day to day (Butte *et al.*, 1988). Therefore, it becomes quite difficult to define a general composition of human milk.

The lactation period is divided into three different stages: colostrum (1-5 days postpartum), transitional milk (6-15 days after birth) and mature milk (after 15 days) (Sala-Vila *et al.*, 2005). The changes in composition are greatest and occur most rapidly during the first week after birth (Emmett & Rogers, 1997). Table 1 shows the basic chemical composition of human milk from healthy women who delivered term infants in the United Kingdom (UK; data from the UK's standard food analysis tables) (Emmett & Rogers, 1997) and Inner Mongolia (China) (Shi *et al.*, 2011) in the three stages of lactation. The nutrient concentrations presented in Table 1 for mature milk are in agreement with other authors (Yamawaki *et al.*, 2005) and The United States Department of Agriculture (USDA) reference values (USDA, 2009).

Table 1. Average basic nutrient composition of human milk in the three lactation stages (g/100 mL, unless otherwise indicated). Adapted from Emmett and Rogers (1997); Shi et al. (2011).

Nutrient	Colostrum		Transitional milk		Mature milk	
	Emmett and Rogers (1997)	Shi et al. (2011)	Emmett and Rogers (1997)	Shi et al. (2011)	Emmett and Rogers (1997)	Shi et al. (2011)
Water	88.2	-	87.4	-	87.1	-
Total solids	-	11.58	-	11.37	-	11.49
Total nitrogen	0.31	1.38	0.23	1.33	0.20	1.27
Non-protein nitrogen	-	0.05	-	0.05	-	0.07
Protein	2.0	1.33	1.5	1.28	1.3	1.20
Lipid	2.6	3.45	3.7	3.29	4.1	3.04
Carbohydrate	6.6	-	6.9	-	7.2	-
Lactose	-	6.79	-	6.65	-	6.97
Energy [a]	56	-	67	-	69	-
Ash	-	0.17	-	0.20	-	0.21

[a] (kcal/100 mL); "-" means no available data.

2.1. Proteins

Protein and total nitrogen contents are higher in colostral milk than in transitional and mature milk (see Table 1). Milk proteins are of two major types: casein and whey proteins, and, unlike cow's milk, approximately two-thirds of the proteins in human milk are whey proteins. The most representative ones are presented in Table 2. Among them the most abundant are α-lactalbumin, lactoferrin and secretory immunoglobulin A (sIgA) (Nagasawa *et al.*, 1973). The higher protein concentration in colostrum is mainly due to the higher concentration of sIgA, but the concentrations of IgM and IgG are also higher in colostral milk than in transitional and mature ones (Table 2). The concentrations of α-lactalbumin and lactoferrin both in colostral and transitional milk are higher than in mature milk (Shi *et al.*, 2011).

Table 2. Main whey protein composition of human milk in the three lactation stages (mg/mL) (Shi *et al.*, 2011).

Protein	Colostrum	Transitional milk	Mature milk
α-lactalbumin	1.38	1.33	1.27
Lactoferrin	3.04	3.01	2.05
Serum albumin	0.27	0.30	0.19
IgA	1.48	1.07	0.33
IgG	0.46	0.22	0.19
IgM	0.12	0.09	0.08

Some proteins in the human milk, such as α-lactalbumin, lactoferrin and casein, are synthesised by the mammary gland whereas others, such as serum albumin, are derived from the mother's blood (Lönnerdal *et al.*, 1976). The concentration of those secreted by the mammary gland decreases during the first days of lactation, while those derived from the mother's blood remain fairly constant (Emmett & Rogers, 1997). Igs, lactoferrin and α-lactalbumin, as well as other proteins with antimicrobial activity (e.g. lysozyme and lactoperoxidase), are relatively resistant to proteolysis in the gastrointestinal tract and contribute to the defence of breastfed infants against pathogenic bacteria and viruses (Lönnerdal, 2003). In agreement with this, breastfed infants have shown higher levels of sIgA in saliva than bottle-fed infants (Uruakpa *et al.*, 2002). Besides, lactoferrin, for example, protects against cancer development and metastasis (Rodrigues *et al.*, 2009).

Non-protein nitrogen (NPN) concentration does not vary significantly during the lactation process (Table 1), because NPN is derived mainly from the mother's blood (Lönnerdal *et al.*, 1976). Functional roles of some NPN containing compounds such as taurine and other free aminoacids, L-carnitine and free nucleotides are not still clear. Yet it is thought that their biological significance is related to the infant growth and development (Ferreira, 2003). Free amino acids are

present in human milk in higher number and concentration than in cows' milk, supporting the previous explanation.

2.2. Lipids

Fat is the main source of energy in human milk and it is distinct from that in the milk of other animals and generally better absorbed by the infant's gut (Emmett & Rogers, 1997). In what concerns lipid content of human milk the results available in literature are contradictory. Emmett and Rogers (1997) observed that the total fat content of breast milk in UK women increases from colostrum to transitional and mature milk, while recently Shi *et al.* (2011) found no significant difference in the total fat between the milk of Inner Mongolia women in the three lactation stages (Table 1). This can happen due to several factors, as fat content of breast milk varies between feeds, depending on the extent to which the breast was emptied during the previous feed, and along a single feed. Lipids appear to be the most variable macronutrient inter- and intra-individuals and with maternal nutrition (Emmett & Rogers, 1997). Regarding the effect of maternal nutrition, it was demonstrated that weight gain during pregnancy was directly related to higher fat concentration in breast milk (Michaelsen *et al.*, 1994). Furthermore, the pattern of fatty acids in maternal diet influences fatty acid composition of her milk (Emmett & Rogers, 1997). Table 3 shows the fatty acid composition of English and Chinese women's milk along lactation.

Fatty acids are the main components of milk fat and, while Shi *et al.* (2011) found no significant variation of these compounds during the three lactation stages, Emmett and Rogers (1997) presented an increasing tendency along the lactation period, mainly for saturated and monounsaturated fatty acids. These differences are probably explained by the intra- and inter-individual fat variability described above. In the case of saturated fatty acids, for example, there were authors who obtained results similar to those found by Shi *et al.* (2011) (Silva *et al.*, 2005), whereas others obtained higher concentration values (Sala-Vila *et al.*, 2005).

Table 3. Average fatty acid composition of human milk in the three lactation stages (%, unless otherwise indicated).

Fatty acid	Colostrum		Transitional milk		Mature milk	
	Emmett and Rogers (1997)	Shi et al. (2011)	Emmett and Rogers (1997)	Shi et al. (2011)	Emmett and Rogers (1997)	Shi et al. (2011)
C10:0	-	1.17	-	1.04	-	0.98
C12:0	-	4.55	-	4.43	-	4.15
C14:0	-	4.82	-	4.81	-	4.60
C16:0	-	21.95	-	22.94	-	24.38
C18:0	-	5.63	-	6.03	-	5.97
C20:0	-	0.12	-	0.13	-	0.13
C22:0	-	0.12	-	0.10	-	0.14
Total saturated	1.1[a]	38.36	1.5[a]	39.48	1.8[a]	40.35
C14:1	-	Tr.	-	Tr.	-	0.24
C16:1	-	3.05	-	3.29	-	3.33
C18:1	-	30.27	-	33.16	-	30.92
C20:1	-	0.60	-	0.76	-	0.57
C22:1	-	0.70	-	Tr.	-	0.22
Total MUFA	1.1[a]	34.62	1.5[a]	37.21	1.6[a]	34.65
C18:2	-	18.88	-	16.51	-	16.97
C18:3 γ	-	0.37	-	0.21	-	1.08
C18:3 α	-	5.27	-	3.39	-	3.29
C20:2	-	0.35	-	0.36	-	0.37
Total PUFA	0.3[a]	24.87	0.5[a]	20.47	0.5[a]	21.71
Cholesterol	31[b]	-	24[b]	-	16[b]	-

[a] (g/100 mL); [b] (mg/100 mL); "-" means no available data; Tr. means trace amounts.

Among other fatty acids, human milk provides all of the dietary essential fatty acids, such as linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3), as well as other longer-chain more-unsaturated metabolites, including arachidonic acid (20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) that support the growth and development of breastfed infants (Innis, 2007b). Shi *et al.* (2011) found a ratio of LA to ALA of 5.2:1 which is in the range of 5:1 to 15:1 recommended by ESPGHAN (European Society of Paediatric Gastroenterology, Hepatology and Nutrition) committee on nutrition. However, these authors did not detect arachidonic acid and DHA in the milk of Chinese women, which were detected by other authors in Brazilian and Spanish mothers (Sala-Vila *et al.*, 2005; Silva *et al.*, 2005).

DHA is of great importance, because it is accumulated specifically in the membrane lipids of the brain and retina where it plays critical roles in the visual and neural systems, including protection from oxidative damage, neurogenesis, neurotransmitter metabolism, membrane protein functions, transmission of the visual signal and regulation of gene expression (Innis, 2007a).

Archidonic acid and DHA are not essential in adult diets, but preterm and very young infants cannot synthesise them fast enough to keep up with their nutritional needs (Emmett & Rogers, 1997). LA and ALA cannot be formed by mammalian cells, so they are secreted in milk as a result of the maternal diet (Innis, 2007b). Most infant formulae do not contain the whole range of fatty acids present in human milk and are low in some, particularly in long chain poly-unsaturated fatty acids (PUFAs) (Emmett & Rogers, 1997).

2.3. Carbohydrates

The concentrations of total carbohydrates and lactose do not vary significantly between colostral, transitional and mature milk (Table 1). Lactose is the main carbohydrate in human milk (≈ 7 g/100 mL) and its concentration seems to be fairly insensitive to changes in diet and nutritional status (Emmett & Rogers, 1997). In newborns lactose is not entirely hydrolysed in the intestine and the small amounts of unhydrolysed lactose that reach the colon are consumed by bacteria, resulting in the preferential growth of bifidobacteria and lactobacilli (Coppa *et al.*, 2006). Besides lactose, oligosaccharides are also present in substantial amounts in colostrum (mean of 2.1 g/100 mL on day 4) and mature milk (mean of 1.3 g/100 mL on day 120) (Coppa *et al.*, 1993). Oligosaccharides, together with proteins, are the third major components of human milk from a quantitative point of view, right after lactose and lipids (Zivkovic *et al.*, 2011).

The prebiotic role of oligosaccharides has been thoroughly described in literature and it is currently undoubted that they promote a bifidobacteria-dominant microflora, contributing to the healthy growth of infants. Several studies outline that they resist digestion and reach the colon where they stimulate the development of the bifidus-predominant flora (Coppa *et al.*, 2006). As only trace amounts of oligosaccharides are present in cows' mature milk (J. A. Lane *et al.*, 2010) and, consequently, in cow's milk-based infant formula, breastfed infants' microflora shows a predominance of bifidobacteria and lactobacilli (90%) (Harmsen *et al.*, 2000), while formula-fed infants' intestinal flora has a significantly lower number of these bacteria (40-60%), with the remaining composed of *Enterobacteriaceae* and *Bacterioides* (Harmsen *et al.*, 2000; Rubaltelli *et al.*, 1998). In a lesser extent, monosaccharides, mostly glucose and fuccose, are present too (Emmett & Rogers, 1997).

2.4. Vitamins

Vitamins, as well as minerals and trace elements, have a high level of bioavailability. Therefore, even at low concentrations they can be well utilised (Lönnerdal, 1985). Maternal milk should supply all of the vitamins' requirements, but vitamin content may vary significantly according to the maternal diet and, specifically, to vitamin dietary intake. Table 4 gathers the breast

milk vitamins' composition of women who gave birth in the UK and in China, two countries with different dietary habits.

Table 4. Average vitamin composition of human milk in the three lactation stages (mg/100 mL, unless otherwise indicated).

Vitamin	Colostrum		Transitional milk		Mature milk	
	Emmett and Rogers (1997)	Shi et al. (2011)	Emmett and Rogers (1997)	Shi et al. (2011)	Emmett and Rogers (1997)	Shi et al. (2011)
Vitamin C	7	1.7[a]	6	2.2[a]	4	1.6[a]
Folate	2[b]	4.7[c]	3[b]	4.6[c]	5[b]	2.4[c]
Pantothenate	0.12	204.5[c]	0.20	186.0[c]	0.25	249.3[c]
Biotin [b]	Tr.	-	0.2	-	0.7	-
Niacin	0.1	180.0[c]	0.1	173.8[c]	0.2	182.7[c]
Riboflavin	0.03	16.9[c]	0.03	17.6[c]	0.03	13.7[c]
Thiamin	Tr.	6.5[c]	0.01	5.2[c]	0.02	6.3[c]
Vitamin B_6	Tr.	5.4[c]	Tr.	4.5[c]	0.01	4.6[c]
Vitamin B_{12} [b]	0.1	-	Tr.	-	Tr.	-
Retinol	155[b]	24.1[d]	85[b]	31.9[d]	58[b]	20.4[d]
Vitamin D	-	159.7[e]	-	97.6[e]	0.04[b]	0.2[e]
Vitamin E	1.30	294.4[c]	0.48	174.5[c]	0.34	234.6[c]
Vitamin K [c]	-	22.4	-	22.7	-	0.8

[a] (mg/100 g); [b] (µg/100 mL); [c] (µg/100 g); [d] (IU/100 g); [e] (IU/100 g^{-1}); "-" means no available data; Tr. means trace amounts.

Water-soluble vitamins' concentrations are usually more affected by maternal diet than the concentrations of fat-soluble vitamins (Prentice *et al.*, 1983). Emmett and Rogers (1997) reported that soluble vitamins as thiamine, niacin, vitamin B_6, folate, pantothenate and biotin increase in concentration from colostrum to mature milk, while vitamin B_{12} and vitamin C decrease and riboflavin concentration remains the same. However, even though the similarity of the results, Shi *et al.* (2011) found that water-soluble vitamins, such as thiamin, niacin, vitamin B_6, folate, pantothenate, riboflavin and vitamin C did not vary significantly along lactation. These differences may be explained by variations in the maternal diet. The concentration of vitamin C throughout lactation was, in the most recent work, lower than the concentration presented by Emmett and Rogers (1997). This is probably due to some traditional Chinese dietary habits; many mothers exclude most fruits and vegetables from their diet during 15 to 30 days after parturition, leading to a great decrease of vitamin C (Shi *et al.*, 2011). In general, the results of both works were similar to the USDA reference values (USDA, 2009).

Among fat-soluble vitamins, those that are most relevant in human milk are vitamin A or retinol and vitamins D, K and E. Emmett and Rogers (1997) observed a decrease in the human milk

content of vitamins A and E over the course of lactation. On the other hand, Shi *et al.* (2011) found a major decrease of vitamins D and K from colostrum to mature human milk. Vitamin A concentration varies according to the mother's diet in pregnancy and lactation, that is, according to the vitamin A status of the mother (Mello-Neto *et al.*, 2009). Thus, lower retinol concentrations found in Chinese women may be associated with maternal food choice in that region (more eggs and rice) (Shi *et al.*, 2011). Regarding vitamins D and K, human milk contains small amounts of these vitamins. Nevertheless, they are important in the prevention of some diseases (Ala-Houhala, 1985; P. A. Lane & Hathaway, 1985). Although there is little evidence of a relation between the mother's diet and vitamin E concentration in breast milk (Emmett & Rogers, 1997), vitamin E concentration in Chinese mothers was lower than reference values (Emmett & Rogers, 1997; USDA, 2009).

2.5. Minerals

The bioavailability of most minerals in human milk is much higher than in cow's milk or infant formula (Emmett & Rogers, 1997), so that they can be efficiently used by the organism as they are present in milk in low concentrations. Results from both studies presented in Table 5 show similar concentrations of calcium (Ca), phosphorous (P), chlorine (Cl; except for mature milk), magnesium (Mg), cooper (Cu), iron (Fe), zinc (Zn; except for colostrum) and selenium (Se). However, Shi *et al.* (2011) obtained lower concentrations of potassium (K) and sodium (Na) and higher concentrations of iodine (I), than those presented by Emmett and Rogers (1997). Other studies that measured mineral and trace element composition of breast milk (Almeida *et al.*, 2008; Yamawaki *et al.*, 2005) showed similar concentrations to those obtained in the works used to create Table 5 (Emmett & Rogers, 1997; Shi *et al.*, 2011).

Table 5. Average mineral composition of human milk in the three lactation stages (mg/100 mL, unless otherwise indicated).

Mineral	Colostrum		Transitional milk		Mature milk	
	Emmett and Rogers (1997)	Shi et al. (2011)	Emmett and Rogers (1997)	Shi et al. (2011)	Emmett and Rogers (1997)	Shi et al. (2011)
Ca	28	28.5	25	28.0	34	33.4
P	14	14.1	16	15	15	16.7
Cl	-	84.1	86	89.7	42	88.8
Mg	3	3.5	3	3.6	3	3.7
K	70	46.0	57	46.3	58	50.4
Na	47	11.0	30	16.3	15	13.8
Cu	0.05	61.3[a]	0.04	59.0[a]	0.04	36.7[a]
Fe	0.07	0.05	0.07	0.05	0.07	0.05
Zn	0.6	0.3	0.3	0.3	0.3	0.2
I [a]	-	22.1	-	33.1	7	27.9
Se [a]	-	1.4	2	1.9	1	1.5

[a] (µg/100 mL); "-" means no available data.

The observed differences are probably due to factors included in the intra- and inter-individual variation, such as maternal diet or nutritional status. The effect of maternal diet in the mineral content of human milk is not the same for all minerals, yet depends on which mineral is being considered. Maternal intake of certain minerals like calcium, magnesium, cooper and zinc does not seem to affect the breast milk concentration (Aggett, 1994; Garg *et al.*, 1988). On the other hand, there is evidence that selenium concentrations in breast milk can be affected by maternal diet (Funk *et al.*, 1990).

3. Human milk banks

As previously stated, it's undoubted that breastfeeding is the ideal way to nourish both term and preterm infants (Dewey, 2001; Morales & Schanler, 2007). Unfortunately, sometimes mother's milk is unavailable or in short supply and the infant cannot be breastfed (or at least exclusively) (Bertino *et al.*, 2009). What happens then if the mother is unable to meet the needs of her baby? In this situation human milk banks (HMB) are essential, as they provide donor milk, which is a better alternative than infant formula (Leaf & Winterson, 2009). Even artificial formula manufacturers have already acknowledge that "Formula or cow's milk is low in functional components and can never be fortified to match breast milk" (Yeung & Peters, 2001). In this sense, the enormous volumes of human milk discarded by mothers who produce more than their infants require may become an invaluable resource if donated to HMB (Hartmann *et al.*, 2007).

Human milk banking consists in collecting, processing and storing human milk from lactating women with the aim of feeding it to other women's babies. This is not a new concept; wet-nursing has been carried out for centuries and since 1909, when the first milk bank appeared, milk banking has been practised all around the world (Leaf & Winterson, 2009). Later on in the 1900's the development and assertive marketing of bovine infant formulas, as well as the emergence of HIV and the fear of other viruses and illness, hospital budget crisis and progress in the neonatal intensive care changed this situation, leading to closings of many banks (Jones, 2003; Leaf & Winterson, 2009). Nowadays, with increasing awareness of donor human milk benefits and safety, the interest in HMB is resurging along with the required resources (Tully *et al.*, 2004).

HMB are expanding in number and capacity and are particularly focused in providing human milk to extremely premature (gestational age below 28 weeks), premature (28 – 33 weeks) or ill hospitalised infants, as they need for larger amounts of protein and energy than the healthy term ones to achieve appropriate growth (Sauer, 2007). Thus, whenever possible, the milk of mothers who give birth to extremely premature or premature infants (extremely preterm and preterm milk, respectively) is preferred, because of its higher contents of protein, fat, carbohydrates and energy (Bauer & Gerss, 2011). Nevertheless, processing and storage of donor milk, as well as mothers' own milk, affect some of its nutritional and immunological properties (Wight, 2001).

In the Perron Rotary Express Milk Bank in Australia they analysed the macronutrient composition of pasteurised donor human milk mainly from mothers who gave birth prematurely (Hartmann *et al.*, 2007). Protein, lactose and energy concentrations were similar to those of unpasteurised mature human milk from healthy mothers of term infants reported by other authors (Emmett & Rogers, 1997; Shi *et al.*, 2011). Fat concentration was slightly higher than that obtained by Shi *et al.* (2011) and similar to that reported by Emmett and Rogers (1997). As above referred, it is known that preterm milk has higher macronutrient content than term milk, hence the similar

macronutrient concentrations between pasteurised preterm milk and raw term milk are probably due to losses during human milk pasteurisation.

Figure 1 represents the WHO hierarchy of feeding choices for low-birth-weight infants (Arnold, 2002), which is very similar to that for regular weight babies that only do not usually require preterm milk. Breastfeeding is always the number one option, but for premature or sick infants who cannot be breastfed donated fresh preterm milk becomes the best option and donated fresh term mature milk the second best. Pasteurised donated breast milk is the number four in WHO choices, because pasteurisation diminishes the nutritional value of human milk and partially destroys immunological and functional factors, as described further in section "Effects of pasteurisation, freezing/thawing and storage". Preterm and ordinary formula come only as the last choices (numbers five and six, respectively).

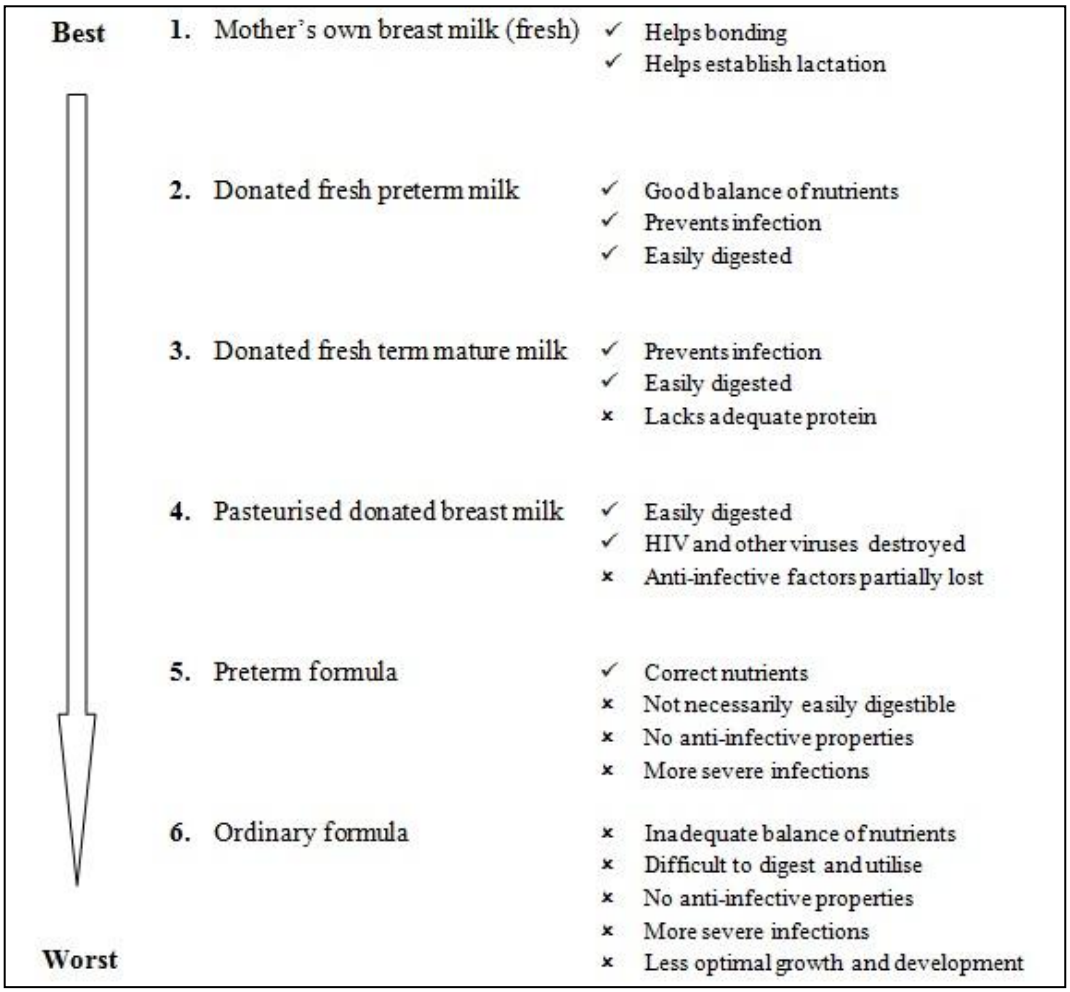

Figure 1. WHO hierarchy of infant feeding choices for low-birth-weight infants. Adapted from Arnold (2002).

3.1. Human milk banking practices

The number of HMB is increasing day by day. Therefore, in the absence of international or national regulation and recognised guidelines, several countries have developed their own guidelines and quality standards (Baumer, 2004; Dall'Oglio *et al.*, 2009; Hartmann *et al.*, 2007; Melo *et al.*, 2010; Updegrove, 2005). In the UK HMB can voluntarily adopt the standards of practice of the UK Association of Milk Banks (www.ukamb.org), but in Canada, Mexico and the United States (US) the Human Milk Banking Association of North America (www.hmbana.org) has different standards (Modi, 2006). There are few countries, such as Brazil, where governments have specific legislation regulating milk banking and difficulties seem to be greatly reduced (Arnold, 2006). The general milk banking practices will be described and explained, taking as example the milk banks of the Human Milk Banking Association of North America (HMBANA), as some procedures differ between countries. In Figure 2 a simplified scheme of the main practices performed in HMB in order to collect and preserve milk is presented.

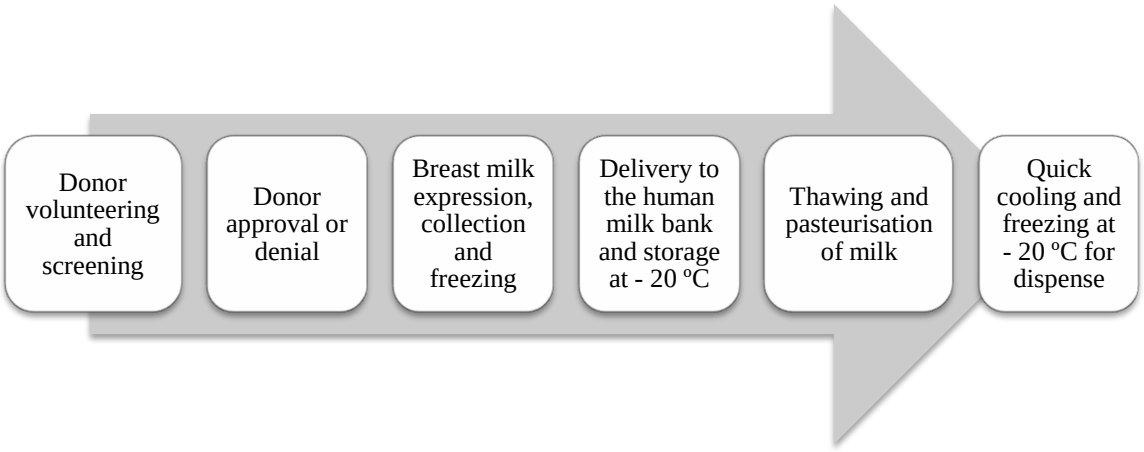

Figure 2. Main practices of the process conducted in HMB.

The whole process starts with the choice of the donors. Donors are self-selected in the first place and are not paid, their satisfaction comes from the fact that they are able to help other babies in need (Updegrove, 2005). After a potential donor manifests the interest in donating her milk the donor screening begins. Firstly, mothers are questioned about mother and baby's health, maternal lifestyle and medical issues. They have to be healthy lactating women willing to donate their surplus milk and must take minimal or no medication and no herbal products (Human Milk Banking Association of North America). Secondly, they are informed about the process of pumping and storing milk and asked to do some blood tests (HIV-1, HIV-2, human T cell lymphotropic virus (HTLV), hepatitis B and C, and syphilis) (Hartmann *et al.*, 2007).

After the blood tests results are known the donors may be approved, denied or asked to answer some more questions, so that a decision can be made (Updegrove, 2005). HMBANA donor exclusion criteria are quite extensive and include any positive blood test among the required ones, risk factors for HIV, use of illegal drugs, use of nicotine products, regular alcohol intake, travelling to the UK for more than 3 months or Europe for more than 5 years since 1980 and travelling or living in Cameroon, Central Africa Republic, Chad, Congo, Equatorial Guinea, Gabon, Niger, or Nigeria (Human Milk Banking Association of North America). The second last criterion is presumably based on the risks of Creutzfeldt-Jakob disease (Modi, 2006) and is not applied in European countries.

Once the donor is approved she has to express the milk, manually or through the use of a breast pump, collect it to disinfected polypropylene bottles, label them and freeze the milk in the coldest part of the freezer (Hartmann *et al.*, 2007). Then, she has to deliver it to the milk bank or other existing collection sites in the area (Updegrove, 2005). In the milk bank, raw donor human milk is stored frozen at – 20 ºC to prevent microbial growth (Pardou *et al.*, 1994) and lipid peroxidation, reduce viable cytomegalovirus (CMV) and preserve vitamin C content (Baumer, 2004). Before being delivered to medically needy individuals or medical institutions, the milk has to be mandatorily pasteurised to inactivate pathogenic microorganisms, part of the commensal flora (Molto-Puigmarti *et al.*, 2011) and also viruses. Currently, the most frequently used method is the in-pack pasteurisation, known in the field as Holder pasteurisation, which is a low-temperature long-time (LTLT) pasteurisation technique. It consists in placing the milk in a water bath and heating it at 62.5 ºC for 30 minutes (Updegrove, 2005).

In order to be pasteurised, the milk is thawed, poured into flasks and mixed carefully to promote homogenisation. Then, a sample of raw milk is taken for microbiology (identification and counting of colonies). Human milk that tests positive for certain bacteria as *Staphylococcus aureus* or any of the bacillus species is discarded. After pasteurisation a second sample is taken for microbiological control. Any bacterial growth in the postpasteurised sample is unacceptable and leads to the discarding of that donor's milk (Updegrove, 2005). It is well known that the germination of spores of bacillus species, for example *Bacillus cereus*, can occur during heat treatment (Hanson *et al.*, 2005). Usually, another sample is collected from the donor's milk for nutritional analysis, before or/and after pasteurisation, depending on which bank is being considered.

In the Mother's Milk Bank at Austin one sample is collected before pasteurisation with the aim of evaluating the nutritional value of human milk and another one after to recheck the nutritional content (Updegrove, 2005). They mix together the milk from two or three donors in certain volumes that allow them to ensure an adequate caloric and protein value for the milk; given

that human milk composition is very variable. At the end they label the bottles, which include nutritional information, and pasteurise the milk. Following pasteurisation the milk is quickly cooled in an ice bath and frozen at – 20 ºC for dispensing (Updegrove, 2005). The whole handling process of human milk in the banks is performed under aseptic conditions.

There are, however, some exceptions to these general procedures. Milk banks in the UK, for example, do not pool donor milk, yet they prepare it in aliquots from individual donors to minimise the risk of multiple donor exposure (Leaf & Winterson, 2009). In some countries like Norway, HMB do not pasteurise donor milk; instead they use raw donor milk to feed preterm infants (Grøvslien & Grønn, 2009). The donors have to be screened for HIV, hepatitis B and C, HTLV and CMV and the milk screened for bacteria to ensure that it is free of pathogens and has low bacterial counts. Also in 5 of the 27 Swedish HMB raw donor milk is used (Omarsdottir *et al.*, 2008).

One of the main constraints of HMB is their financial implications and the high costs of the final product. Even though donors of human milk are not paid, the costs of screening, pasteurisation, storage and transportation are high. Do the benefits justify the costs? Some authors have made simple comparisons in order to assess economical potential of these banks. As recently reviewed by Leaf and Winterson (2009) in UK, costs of transport, pasteurisation, storage, staff training and administration were determined to be up to £150/litre (Tully, 2000). It is much higher than the cost of preterm formula (around £10/litre), but of minor significance when compared to the cost of a neonatal bed (around £189-£355/day in 2000) (O'Neill *et al.*, 2000).

Likewise, Simmer and Hartmann (2009) compared the cost of pasteurised donor human milk with the cost of staying in the neonatal intensive care unit (NICU). In the US, private non-profit milk banks charged about US$3.00 per 30 mL of donor human milk in 2009, which was equivalent to AU$120 per litre at the time. Following Australian NICU standard, they assumed that a hypothetical infant being fed exclusively donor milk for 24 weeks would require 10 L of milk, which would cost around AU$1200. They added that this cost is equivalent to less than a day's care in their NICU. Therefore, if providing donor milk helps prevent some of the complications attributed to artificial formula use, the length of stay in the NICU may be significantly reduced and the investment greatly monetised (Simmer & Hartmann, 2009).

3.2. Effects of pasteurisation, freezing/thawing and storage

Due to the effects of heating, cooling, freezing, and storing, some of the most valued and precious components of human milk are diminished or destroyed, while feeding fresh milk (or at least fresh frozen and not heated) preserves most of the components (Lawrence & Lawrence,

2011). This section will be focused on reviewing the effects of pasteurisation, freezing and thawing and cold storage on human milk.

Even though pasteurisation of donor human milk eliminates the risk of transmission of infectious agents, it does affect some of the nutritional and immunological components of human milk (Wight, 2001), as evidenced by numerous studies. A recent study, which determined the effect of Holder pasteurisation, freezing at – 20 ºC and thawing on fat, protein and lactose concentrations in human milk, found a significant reduction of fat (5.5%) and protein (3.9%) concentrations following these processes (Vieira *et al.*, 2011). It was demonstrated that the major process responsible for these losses was pasteurisation. Lactose concentration did not suffer a significant reduction (0.5%) throughout the processes studied. A previous study where pooled human milk was subjected to Holder pasteurisation and storage at – 20 ºC up to 90 days obtained similar reduction of fats (of 6%) (Lepri *et al.*, 1997). In addition, both of the works are in agreement that freezing and thawing may alter the structure of the fat globule, causing lipolysis of breast milk.

Lepri *et al.* (1997) also reported that pasteurisation induced triglyceride hydrolysis. In this work, the amount of free fatty acids (FFAs) doubled after pasteurisation and rose even more after storage. Wardell *et al.* (1981) found lower percentage of linoleic (C18:2; -22%) and linolenic (C18:3) acids in milk after freezing, thawing and Holder pasteurisation. Another study found that the proportion of medium-chain saturated fats (C12:0, C14:0) was increased after pasteurisation, with a trend towards a decreased proportion of oleic acid (C18:1n-9) (Ewaschuk, Unger, O'Connor, *et al.*, 2011). In contrast, other studies showed no significant differences in human milk fatty acid proportions before and after pasteurisation (Fidler *et al.*, 1998; Henderson *et al.*, 1998; Romeu-Nadal *et al.*, 2008). Nevertheless, a randomised study found that feeding preterm infants with pasteurised milk as compared with raw milk reduced fat absorption in 17%, causing them to gain less weight (Andersson *et al.*, 2007). There is the likelihood that these last results are due to inactivation of milk lipases during pasteurisation, which will be discussed further on, and the consequential removal of the filaments from milk fat globules (Buchheim *et al.*, 1988). Besides these macronutrients, it has been demonstrated that Holder pasteurisation does not affect the concentration and pattern of oligosaccharides in human milk (Bertino *et al.*, 2008).

The most severe effects of Holder pasteurisation on human milk are reported to be in immune factors and certain enzymes. As reviewed by Heiman and Schanler (2007), the main immune factors and enzymes decreased are: sIgA (20 to 50%), total IgA (0 to 50%), lactoferrin (0 to 65%), lysozyme (0 to 65%), lymphocytes (100%), lipase (100%) and alkaline phosphatase (100%) (Baum, 1980; Garza *et al.*, 1986; Hamprecht *et al.*, 2004; Koenig *et al.*, 2005). In addition, it is estimated that 34% of IgG in human milk is degraded by pasteurisation (Evans *et al.*, 1978). Henderson *et al.* (1998) confirmed that the two lipases present in human milk, lipoprotein lipase

(LPL) and bile salt-stimulated lipase (BSSL), were completely inactivated during pasteurisation, while amylase lost about 15% of its initial activity.

Recently, Holder pasteurisation method was shown to structurally modify and decrease BSSL, lactoferrin and components of the immune system and increase lysine bioavailability (Baro *et al.*, 2011). It was also demonstrated that pasteurisation significantly reduces the concentrations of several immunoactive compounds present in the human milk, such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-10 and hepatocyte growth factor (HGF) (Ewaschuk, Unger, O'Connor, *et al.*, 2011). These results are in accordance with a previous study where the authors observed that cytokine concentrations declined following pasteurisation, and suggested that more pro-inflammatory than anti-inflammatory cytokines are retained (Giorgi *et al.*, 2006). Transforming growth factors α (TGF-α) and β_2 (TGF-β_2) are preserved in milk heated at 56.5 ºC for 30 minutes (McPherson & Wagner, 2001).

Vitamin content before and after Holder pasteurisation was investigated as well, and, while vitamins A, D and E were not affected, the concentrations of vitamins C, folacin and B_6 were lowered in 36, 31 and 15%, respectively (Van Zoeren-Grobben *et al.*, 1987). A latest study confirmed that vitamin E did not vary and that pasteurisation significantly decreased vitamin C (20%) and ascorbic acid (16%) levels. (Molto-Puigmarti *et al.*, 2011). There were no changes in the mineral content of milk regarding minerals like Ca, P or Na (Williamson *et al.*, 1978).

Cold storage, freezing and thawing of donor human milk are similar to the practices used by a mother with her own milk for her baby, but these processes may affect human milk composition, too. Garza *et al.* (1986) determined the effects of freezing at – 20 ºC for 3 months in some components of raw human milk. They observed a small decrease in total IgA (3%), but no changes in sIgA or lactoferrin concentrations. Lysozyme concentration was reduced up to 20% and white blood cells were almost completely destroyed. In addition, they verified that storage in the refrigerator at 4 ºC for 24 hours results in several losses: vitamin C (40%), lysozyme (40%), lactoferrin (30%), lipase activity (25%), sIgA (40%), and specific sIgA antibody (0 to 60%). Refrigerated milk showed a reduction of 40% in its phagocytic activity, an increase in the number of cellular elements and also a large increase in FFAs, suggesting spoilage (Garza *et al.*, 1986). Evans *et al.* (1978) reported no appreciable losses of lactoferrin, lysozyme, IgA and IgG after storage by deep freezing at – 20 ºC for 3 months.

We can conclude that collecting, processing, and storing of human milk substantially diminishes its advantages and its most valued properties. Moreover, the loss of several biologically active milk components, mainly due to the heat treatment, is the bottleneck obstructing the diffusion of donor HMB worldwide. Therefore, milk banks should be committed to finding novel

pasteurisation technologies that can assure microbial and viral inactivation, while preserving the nutritional, immunological and functional value of human milk.

4. High-pressure processing

Nowadays, increasingly demanding consumers require not only safe, but also high quality and fresh-like foods. This implies less extreme treatments and fewer or even no additives (Palou *et al.*, 2007). There are several physical (e.g. heating, freezing, dehydration and packaging) and chemical (e.g. acidification and use of preservatives) food preservation methods, but the most commonly used is thermal treatment (e.g. pasteurisation and sterilisation). Even though heat treatment significantly reduces microbial levels and growth, it often results in undesired changes in foods, such as losses of colour, flavour, texture, smell and nutritional value, that is, an overall reduction of freshness and quality of the final product (Hogan *et al.*, 2005). Therefore, new processing technologies are required to provide safe, fresher-tasting, nutritive foods with a reasonable shelf life and improved convenience (Ramirez *et al.*, 2009). High-Pressure Processing (HPP) potentially answers many, if not all, of these challenges (Hogan *et al.*, 2005).

HPP is a novel non-thermal processing technology of great interest in food research and industry, primarily as an alternative to thermal treatment. It consists in subjecting the desired product to pressures that frequently range from 100 to 1000 MPa, i.e., 1000 to 10 000 times atmospheric pressure, and involves the following general steps: placing the product (liquid or solid), with or without packaging, in an adequate vessel and, following closure of the vessel, applying pressure either through a piston or a pump (Huppertz *et al.*, 2006).

The use of high pressure *per se* is not new; this technology has long been applied in various non-food industries, including production of plastics, ceramics, metal-forming and pharmaceutical tablet manufacture (Schaschke, 2011). Even today high pressure is still being studied for other innovative non-food applications (Oliveira *et al.*, 2012; Salvador *et al.*, 2010). HPP effects on foods were first studied in the late nineteenth century, when it was observed that processing milk at 670 MPa for 10 min achieved five to six microbial logarithmic reductions, extending shelf life up to 4 days after processing (Hite, 1899). Even though HPP has been successfully applied in foods for over 100 years, the commercialisation of products processed by HPP is fairly recent (Table 6). This technology took about a century to reach the market mainly due to its high cost, lack of commercial equipment capable of operating with minimal disruptions and processing large amounts of food, and lack of investment in the emerging technologies by the food industry, particularly in Europe (Patterson *et al.*, 2006; Torres & Velazquez, 2005).

Table 6. Remarkable events in the history of HPP for food products. Adapted from Patterson et al. (2006) and supplemented with informations from Food ingredients first (2011); Nguyen et al. (2010); Tonello (2011).

Year	Event(s)
1895	Royer (France) used high pressure to kill bacteria experimentally
1899	Hite (USA) used high pressure for food preservation
1980s	Japan started producing high pressure jams and fruit products
1990s	Fresherized Foods (formerly Avomex; USA) began to produce high pressure guacamole from avocados with a fresh taste and extended shelf life
2000	Mainland Europe began producing and marketing fresh fruit juices (mainly citrus) and delicateness-style cooked meats. High pressure self-sucking oysters, poultry products, fruit juices and other products were marketed in the USA
2001	HPP fruit pieces given approval for sale in the UK. Launch of the first HPP fruit juices in the UK
2003	Espuña (Spain) launched a line of ready-to-microwave HPP meat snacks (e.g. bacon and cheese rolls). In 2005, the company developed the first high pressure sliced cured ham.
2008	Fonterra (New Zeland) developed a pressurised antibody rich colostrum beverage
2009	FDA approval of the pressure-assisted thermal processing (PATP) for production of low acid foods, such as mashed potatoes
2011	Starbucks aquired Evolution Fresh with the aim of bringing premium HPP juices to the marketplace

In the 1980s, the desire for fresh long shelf life products in Japan brought to the market the first HPP product, a fruit jam (Table 6), as these needs could not be fully satisfied by any other existing technology (Patterson *et al.*, 2006). Since then, a number of HPP foods from fruit juices to fish products like raw squid appeared in the Japanese markets (Hayashi, 1992). In the USA, foods such as avocados, oysters and other shellfish, and meat products have greatly benefited from high pressure in terms of fresher taste, extended shelf life and consumer safety (HPP effectively reduced the number of spoilage and pathogenic microorganisms, but preserved the fresh-like characteristics). In Europe and the USA HPP fruit juices were and still are a commercial success, so that the largest coffeehouse company in the world, Starbucks, acquired Evolution Fresh in 2011 in order to enter the Health and Wellness sector by providing premium tasteful juices (Food ingredients first, 2011). In just two decades HPP has clearly stood out from the set of food preservation emergent technologies, as reflected by the increasing number of high pressure units installed over the world (Figure 3), and is now a routine procedure for the processing of several commercialised foods. Current HPP foods on the market are mostly refrigerated or have reduced water activity and/or low pH to prevent bacterial spores germination, as HPP technology alone is limited to pasteurisation treatments (Mújica-Paz *et al.*, 2011).

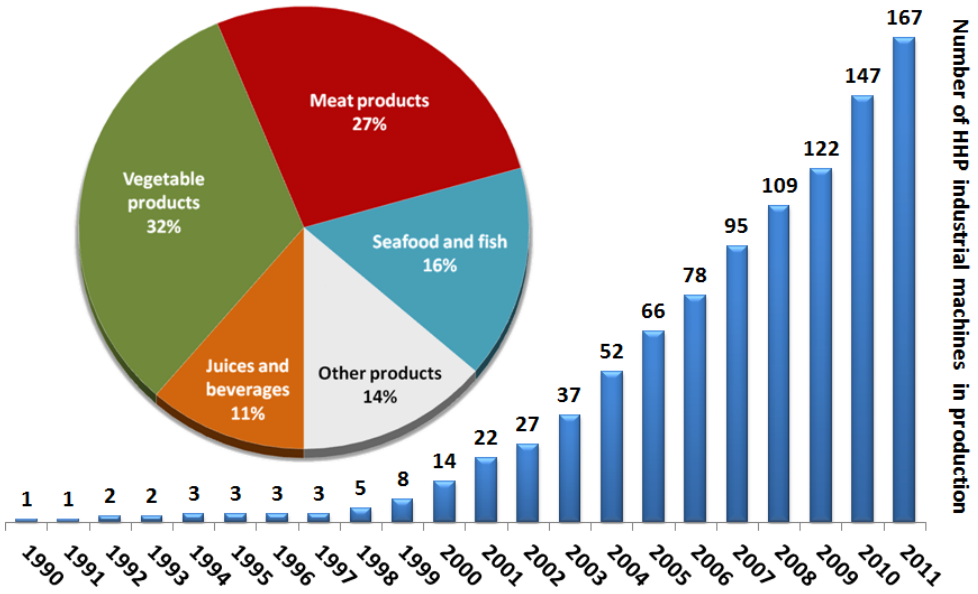

Figure 3. Hiperbaric data of world growth and main applications of HPP in the food industry. Courtesy of Hiperbaric.

As shown in Figure 3, this technology has been used for a wide range of products: vegetables, meats, seafood, fish, fruits, purees and others (e.g. dairy products). In addition, HPP is also increasingly studied for the modifications of functional properties of foods (Correia *et al.*, 2011), and efforts have been made to develop new functional foods for demanding niche markets (Mújica-Paz *et al.*, 2011). A prime example is the fat-free antibody rich colostrum beverage developed by Fonterra in New Zeland (COL+, 2009; Hembry, 2008). Because of its higher price, HPP products are still, yet less and less, targeted for certain niche markets with specific well-defined needs.

The success of new food processing technologies is highly dependent on consumers' acceptance. Regarding consumers acceptance of HPP technology, there are some recent studies reporting that, although the knowledge of what is the "high-pressure processing" is still scarce, HPP has a very positive response and acceptance by the consumers when comparing with the other emerging processing technologies. Besides, when compared to thermally processed foods HPP products have a higher quality, which also contributes for its great consumer acceptance (Wright *et al.*, 2007). Cardello *et al.* (2007) studied consumer perceptions of foods processed by diverse innovative and emerging technologies and concluded that irradiation and genetic modification resulted in the greatest negative response, meeting with consumer resistance, while high-pressure processing produced the most positive effect. On the other hand, Nielsen *et al.* (2009) studied consumer perception of HPP and pulsed electric field (PEF) for processing juice and baby food and verified that consumers perceived the naturalness, improved taste and high nutritional value of HPP

and PEF products, but they lacked information about these technologies. When comparing the two, HPP showed a higher consumer acceptance.

4.1. Principles and operation

It is long known that HPP (at refrigeration, ambient or moderate temperatures) inactivates pathogenic and spoilage microorganisms in foods with fewer changes in colour, flavour and texture than the conventional thermal methods (Cheftel, 1995). In this section the most important principles in which high pressure application effects are based, as well as the general HPP process will be described.

There are two fundamental scientific principles that are applied in the high-pressure processing of foods. The first is based on the Le Chatelier's Principle that states that when a system at equilibrium is disturbed, the system responds in order to minimise the disturbance (Pauling, 1964). According to this, any chemical reaction, conformational change, or phase transition that is accompanied by a decrease in volume will be enhanced by pressure, while reactions involving an increase in volume will be inhibited (Cheftel, 1995). However, due to the complexity of foods and wide variety of phenomena that occur under pressure, it is difficult to predict HPP effects on foods (Palou *et al.*, 2007).

The second is the principle of isostatic processing or the Isostatic Rule. This principle states that high pressure is uniformly and nearly instantaneously transmitted throughout the food (Torres & Velazquez, 2008), whether there is a direct contact with the pressure medium or the food is hermetically sealed in a flexible package that transmits pressure (Olsson, 1995). The food is compressed by uniform pressure from every direction (unlike heat processing where there are temperature gradients) and returns to its original shape when the pressure is released (see Figure 4). Thus, in contrast to thermal processing, the time necessary for pressure processing is independent of equipment and food geometry and size. Therefore, a number of advantages arise from this principle: treatment times can be and usually are short, the surface area of the food does not get over-processed, scaling of laboratory and pilot plant findings to commercial production are both simple and safe, and changes in the equipment or product packaging do not require new pressure and time conditions and process redesign (Mújica-Paz *et al.*, 2011; Schaschke, 2011). None of these advantages is verified in thermal processes.

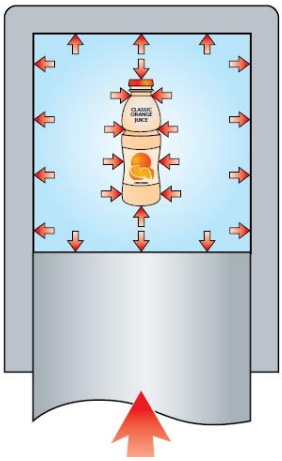

Figure 4. The principle of isostatic processing (Avure Technologies, 2011).

Moreover, when looking at the molecular level, pressure does not affect the covalent bonds of various food components (e.g.

proteins, saccharides, vitamins, lipids and pigments), and sensory properties, nutrients or bioactive compounds suffer no significant losses, again in contrast to the very often highly damaging effects of temperature. Molecular compression is only capable of affecting the weaker bonds and forces, such as hydrogen bridges, electrostatic interactions and van der Walls forces. This is sufficient to alter the delicate molecular structures (e.g. proteins) (Schaschke, 2011) and explains the preservation effect of HPP.

A high pressure system consists of a high pressure vessel and its closure, pressure-generation system, temperature-control device, and material-handling system (Mertens, 1995). Industrial HPP can be a batch or semi-continuous process; solid foods or foods with large solid particles can only be processed in a batch mode, while liquids or slurries can be processed either way (Hogan *et al.*, 2005). Currently, most high pressure machines used in the food industry are batch systems. Figure 5 presents three examples of modern HPP batch units.

Figure 5. HPP units of three companies manufacturing industrial-scale high pressure equipments. Courtesy of Avure Technologies, Uhde High Pressure Technologies and Hiperbaric.

In a batch system, after loading the vessel with the product(s) to be treated and closing it, it is filled with the pressure-transmitting medium, which may be plain water (as in industrial application on foods) or other media containing castor oil, silicone oil, sodium benzoate, ethanol or glycol (Hogan *et al.*, 2005). Then air is removed from the vessel and high hydrostatic pressure is generated. High pressure is usually generated by direct or indirect compression, that is, either by reducing the volume of the pressure chamber inside the vessel using a piston for example or by pumping medium into the vessel, respectively (Palou *et al.*, 2007). When the desired pressure is reached the pump or piston is stopped and the pressure is maintained without further energy use. At the end of the hold time the system is depressurised, the vessel opened and the product unloaded

(Hogan *et al.*, 2005). The time spent for pressurisation, holding and depressurisation is called 'cycle time'.

As noted above, the pressures commonly used range from 100 to 1000 MPa (industrial application in foods usually uses a maximum of 500 MPa). Physical compression during HPP increases the temperature of foods by approximately 2 to 3 °C per 100 MPa, because of the adiabatic heating (Balasubramanian & Balasubramaniam, 2003). However, it is dependent of food composition; foods with high fat content have higher temperature increases. Moreover, during HPP there is a displacement of the equilibrium associated with food pH, typically towards more acid values, contributing to microbial inactivation (Mathys *et al.*, 2008). Once the pressure is release initial pH value is re-established.

In batch systems the food product is generally treated in the final package to assure its maximum security (primarily against microbial contamination) until opening by the consumer. As the volume of foods decreases with compression and expands with decompression, HPP requires hermetic packages that can withstand a change of volume corresponding to the compressibility of the product (Hugas *et al.*, 2002), without compromising seal integrity. Food compression is about 15% for a 600 MPa treatment, reflecting mostly the compression of its moisture content, but it increases if the food has empty spaces or high-fat content (Mújica-Paz *et al.*, 2011). The compression of the headspace increases pressurisation time and consequently processing costs and it is obviously uneconomical to treat empty spaces. Thus, vacuum-packed products are ideal for HPP. Generally, plastic packages are also suitable, while metal cans and glassware are not (Hogan *et al.*, 2005).

5. High-pressure processing of human milk

HPP is the only emergent processing technology that has reached the markets of several countries with a variety of new products, because of its great advantages and consumer's acceptance. However, regarding human milk there are only few reports on the effects of high pressure on its nutritional and microbial contents. Knowledge and fundamental understanding of pressure maintenance of macronutrients and immunological and functional agents, as well as inactivation of microorganisms and enzymes in human milk is still largely lacking. Thus, extensive investigation is required if one wants to make this a reliable alternative to the conventional Holder pasteurisation. To our knowledge, only four studies assessed HPP effects on human milk so far, those indicated in Table 7, where pressure application conditions and main results obtained are summarised.

Table 7. Studies of HPP effects on diverse mature human milk components, including bacterial contamination.

Reference	Components	HP Treatment	Conclusions
Viazis *et al.* (2007)	Immunoglobulin A, lysozyme activity	400 MPa for 30 to 120 min at 21 to 31 °C	HPP samples retained significantly higher levels of IgA and lysozyme activity than Holder's
Viazis *et al.* (2008)	Inactivation of five selected bacterial pathogens	400 MPa for 0 to 50 min at 21 to 31 °C	Effective in reducing the bacterial population; *E. coli* and *S. aureus* ATCC 6538 are the most pressure-resistant (6-$\log_{10}$ reduction after 30 min)
Permanyer *et al.* (2010)	Immunoglobulin A, bacterial load	400, 500 or 600 MPa for 5 min at 12 °C	400, 500 and 600 MPa: IgA retention of 100, 87.9 and 69.3% (Holder: 72%); reduction of bacteria to undetectable levels
Molto-Puigmarti *et al.* (2011)	Vitamin C, fatty acids and tocopherols	400, 500 or 600 MPa for 5 min at 12 °C	Fatty acids and tocopherol did not vary with HPP and Holder, vitamin C was maintained after HPP (Holder: - 20%)

The authors of these studies also performed Holder pasteurisation in order to compare the results obtained by both processing technologies. The experimental conditions and results of these studies will be presented in more detail in the following discussion topics. It is noteworthy that all the four analysed mature human milk from mothers who gave birth at term, so there is not any published work investigating HPP effects neither on colostrum and transitional milk nor on preterm milk. Term milk in the early lactation stages and preterm milk are nutritionally richer than mature term milk, so also in this area more research is needed.

5.1. HPP effects on human milk components

Total protein, fat or carbohydrate contents after HPP have not been determined. There are also no studies concerning HPP effects on lactose, oligosaccharides or minerals and only one whey protein, one enzyme, two vitamins and thirty-eight fatty acids have been studied. As presented in Table 7, the human milk components that have been analysed before and after HPP are total IgA, lysozyme, vitamin C, tocopherols and fatty acids.

5.1.1. Whey proteins and indigenous enzymes

Viazis *et al.* (2007) were the first ones to study high-pressure processing of human milk. They investigated the effects of HPP on total IgA and lysozyme activity in comparison with Holder (or LTLT) pasteurisation. These two proteins have a remarkable antimicrobial activity and are significantly reduced after heating. Milk samples were processed at 400 MPa at 21 to 31 °C for 30, 60, 90 and 120 min (HPP) or at 62.5 °C for 30 minutes (Holder). HPP samples retained 85.6, 87.1, 80.6, and 75.4% of total IgA activity respectively, while LTLT pasteurised milk retained 51.2% activity. For lysozyme, pressurised human milk retained 106.9, 96.3, 96.3, and 95.8% activity respectively, while LTLT pasteurised milk retained 78.8% activity. These promising results indicated that HPP could be a better choice than Holder pasteurisation for pasteurising human milk. Even with treatments four times longer, HPP resulted in a higher maintenance of the both proteins; after 120 min of HPP lysozyme activity decreased by 4.2%, but after 30 min of LTLT pasteurisation its activity decreased 21.2%. For lysozyme even an increase in activity following treatment for 30 minutes (106.9% retention) was observed.

Later on, Permanyer *et al.* (2010) studied the effect of HPP treatments on human milk IgA retention at equal or higher pressure levels for a shorter time. These authors pressure processed human milk at 400, 500 and 600 MPa for 5 min at 12 ºC and determined IgA retention following the treatments, comparing it with the retention after Holder pasteurisation. It was observed that HPP samples treated at 400, 500 and 600 MPa retained 100, 87.9, and 69.3% of IgA respectively, and low-temperature long-time pasteurised ones retained 72%. These findings suggest that HPP at 400 MPa for 5 min at 12°C maintains the immunological protective activity associated with IgA antibodies, and even the pressure treatment at 500 MPa has higher IgA retention that the LTLT method.

Both of the works give valuable information about HPP capacity to better retain proteins with great significance for the infant's health (pressures ≤ 500 MPa). Currently, the only enzyme that has been analysed after HPP of human milk is lysozyme, but studies on the HPP of bovine milk reveal that most indigenous milk enzymes are quite baroresistant. As reviewed by Huppertz *et al.* (2006), most milk enzymes like plasmin, alkaline phosphatase, lactoperoxidase, xanthine

oxidase, phosphoisomerase, γ-glutamyltransferase and lipase are resistant to pressures up to 400 MPa.

High pressure effects on proteins (including enzymes) vary extensively with: the protein being considered; the pressure, temperature and time of the processing; and the pH, and composition of the food to be treated (Mújica-Paz *et al.*, 2011). As pressure-treated proteins maintain their primary structure (because covalent bonds are not affected by HPP), the largest contribution to protein/enzyme inactivation comes from structural rearrangements of proteins under pressure. At pressures higher than 200 MPa changes in the tertiary structure of proteins may occur and are due to dissociation of hydrophobic and ionic interactions (Balny & Masson, 1993).

5.1.2. Fatty acids

Fat content of pressurised human milk has not been assessed yet. However, Molto-Puigmarti *et al.* (2011) recently performed the analysis of thirty-eight fatty acids (from C8:0 to C22:6 n3) in human milk subjected to three HPP treatments and Holder pasteurisation. HPP treatments consisted in processing milk at 400, 500 and 600 MPa for 5 min at initial temperature of 21 °C. No statistically significant differences in the proportions of fatty acids were detected between pressurised, thermally pasteurised and untreated samples. Besides, pressure did not cause significant isomerisation of conjugated linoleic acid (CLA) in human milk. This indicates that infants receiving donor human milk treated by either of the two methods would not be deprived of fatty acids in general.

The authors could not guarantee that pressurisation was able to maintain the triglyceride structure intact, as HPP stability of human milk lipases has not been studied yet and the performed analysis quantified both fatty acids in lipid structures and free fatty acids (Molto-Puigmarti *et al.*, 2011). There is evidence that LPL in bovine milk is quite resistant to high pressures (Pandey & Ramaswamy, 2004; Seyderhelm *et al.*, 1996). In accordance with the described work, no changes in the proportions of fatty acids and CLA were found in high pressure homogenised cow's, ewe's and goat's milks (Rodríguez-Alcalá *et al.*, 2009).

5.1.3. Vitamins

Molto-Puigmarti *et al.* (2011) studied HPP and Holder pasteurisation of vitamins C and E in human milk. The effects of pressure processing at 400, 500 and 600 MPa for 5 min (at 12 °C) were compared to those of thermal pasteurisation at 62.5 °C for 30 min. In terms of vitamin C, they determined the total vitamin C and ascorbic acid contents, and for vitamin E three tocopherol isomers were quantified: delta-, gamma- and alpha-tocopherols (Molto-Puigmarti *et al.*, 2011). No changes in the vitamin C or the ascorbic acid contents of milk were found after HPP, while Holder

pasteurisation decreased them in 19.9 and 16.2%, respectively. The total vitamin C and ascorbic acid retentions were the same in the three HPP treatments. Regarding vitamin E, neither HPP nor Holder pasteurisation caused a significant decrease in the three tocopherols levels. These findings that HPP maintains vitamin C and tocopherol levels in human milk under the tested conditions agree with the idea that small molecules with no higher levels of structure, such as vitamins, are minimally or not affected by high pressure when samples are treated at mild temperatures (Balci & Wilbey, 1999).

5.2. HPP effects on bacteria in human milk

There are only two studies that assess HPP effects on vegetative bacteria present in human milk and both of them are very recent; the first investigated the efficacy of HPP for inactivation of five selected pathogens (Viazis *et al.*, 2008), while the second evaluated HPP effects on total bacterial population in general and bacteria of the *Enterobacteriaceae* family in particular (Permanyer *et al.*, 2010).

Viazis *et al.* (2008) chose the following pathogenic bacteria: *Listeria monocytogenes* ATCC 19115, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and ATCC 6538, and *Streptococcus agalactiae* ATCC 12927. They inoculated pooled donor human milk with each pathogen (10^8-10^9 CFU/mL) and subjected each one of them to a pressure treatment of 400 MPa at 21 to 31 °C for holding times that ranged from 0 to 50 min, depending on the organism being treated. As in the previously mentioned studies, the results of this treatment were compared with those obtained after Holder pasteurisation. Holder pasteurisation resulted in a complete inactivation of all of the pathogens in human milk after 10 min. With respect to HPP, treatments of 2 and 4 min resulted in a complete inactivation of *L. monocytogenes* and *Strep. agalactiae* in human milk, respectively. However, the other pathogens were more difficult to inactivate. After 30 min of pressure treatment *Staphylococcus aureus* ATCC 25923 achieved an 8-$\log_{10}$ reduction and *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* were both reduced by 6-$\log_{10}$. These data suggest that, even though Holder pasteurisation achieved higher levels of bacterial reduction in a shorter time for *Escherichia coli* and *Staphylococcus aureus* ATCC 25923 and ATCC 6538, HPP may be a viable alternative for pasteurisation of human milk. The authors highlight that it cannot be forgotten that HPP results in an overall improved nutritional quality of pasteurised human milk.

More recently, Permanyer *et al.* (2010) studied the bacterial load of human milk after HPP and LTLT pasteurisation, recording total bacteria and *Enterobacteriaceae* counts. Raw milk samples presented a total bacterial count ranging from 1.3×10^2 to 2.9×10^4 CFU/mL and only one sample had a significant *Enterobacteriaceae* count of 3.0×10^1 CFU/mL. These samples were subjected to three HPP treatments of 400, 500 and 600 MPa for 5 min at 12 °C. The three HPP

treatments reduced total bacterial and *Enterobacteriaceae* counts to undetectable levels, regardless of the initial bacterial load, as did LTLT pasteurisation.

When comparing HPP treatment at 400 MPa in both works, although temperature is variable, it is possible to observe that the time necessary for vegetative bacteria inactivation is highly dependent of the initial contamination level (U.S. Food and Drug Administration), showing the importance of milk being collected and stored by the mothers in hygienic conditions and being handled in aseptic conditions in the HMB. Of the diverse bacterial communities in healthy human milk, the most abundant genera are *Streptococcus*, *Staphylococcus*, *Serratia*, *Pseudomonas* and *Corynebacteria* (Hunt *et al.*, 2011), so more studies of HPP effects on these bacteria should be conducted, as they may (above certain levels) cause infections and diseases or, mainly in the case of *Pseudomonas* species, milk spoilage. However, several studies regarding HPP of bovine milk appear to indicate that Gram-positive species such as *L. monocytogenes* and *S. aureus* and Gram-negative *E. coli* are the most baroresistant of the investigated species (Huppertz *et al.*, 2006) and HPP was able to effectively inactivate these pathogens in human milk (Viazis *et al.*, 2008).

There is not any paper describing high pressure effects on bacterial spores, viruses, fungi or prions in human milk, only vegetative bacteria have been analysed. Thus, further research is needed to evaluate the efficacy of HPP in the inactivation of these potential hazards for the babies' health, mainly relevant viral pathogens and spore-forming bacteria.

6. Conclusions

Human milk has proven to provide the most appropriate nutritional, immunological and functional support for newborn infants. Besides the role that breastfeeding plays in the regular development of the infant, it also protects against infectious diseases in infancy and childhood. The composition of human milk is not homogeneous over the course of lactation and colostrum is particularly rich in proteins with immunological function. Furthermore, maternal diet is a key factor affecting human milk composition.

When the mothers cannot adequately supply their children, donated fresh milk is the recommended nutrition by the World Health Organization (WHO), being followed by pasteurised donated breast milk, preterm formula and ordinary formula as the last option. Therefore, human milk banks (HMB) are of an extreme importance for newborns and infants that cannot be breastfed. Nevertheless, currently applied Holder pasteurisation is primarily responsible for a number of losses of bioactive compounds and immune factors, significantly decreasing the benefits of mother's own milk.

According to the published studies on the high-pressure processing (HPP) effects in human milk, HPP has the potential to be developed into a successful pasteurisation method for human milk, as it allows efficient inactivation of the studied microbial pathogens, while maintaining unique components that Holder pasteurisation diminishes and that are crucial for the healthy growth of neonates, infants and young children. There is, however, few available information describing HPP effects on the nutritional content of human milk. Fundamental knowledge is still lacking, so extensive research is required to make this a reliable alternative to the conventional thermal pasteurisation.

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CHAPTER 2

Effect of Thermal Pasteurisation and High-Pressure Processing on Immunoglobulin Content and Lysozyme and Lactoperoxidase Activity in Human Colostrum

Abstract

Human milk, and particularly human colostrum, is the gold standard for newborn nourishment. Colostrum contains the highest concentration of immune factors, being the most potent immune booster known to science. Human milk banks are essential for providing donor milk to newborns who cannot be breastfed. Holder pasteurisation is commonly applied to assure microbial inactivation, but degrades important antimicrobial components, as immunoglobulins (Igs) and lysozyme. High-pressure processing (HPP) is able to assure microbial safety while preserving functional properties of foods. In this work, we investigated the effects of Holder pasteurisation and HPP in colostral IgA, IgM, IgG, lysozyme and lactoperoxidase. All of the Igs were preserved after HPP at 200 and 400 MPa, and partially lost after pasteurisation (20, 51 and 23% decreases for IgA, IgM and IgG, respectively). HPP at 600 MPa resulted in similar losses to thermal pasteurisation. Lysozyme activity was maintained after HPP and 44% lost after pasteurisation. Lactoperoxidase activity was not detected in quantifiable amounts. As far as the authors are aware, this is the first study evaluating HPP effects on human colostrum.

1. Introduction

Breastfeeding is the recommended nutrition for newborns and infants, since human milk provides nutrients, bioactive components and immune factors. The unique composition of this complex fluid promotes adequate growth and benefits the infants in several ways; e.g. enhancement of host defences and prevention of many infectious diseases, as reviewed in Chapter 1. Human milk composition varies over the course of lactation, so that the lactation period is divided into three different stages: colostrum (1-5 days postpartum), transitional milk (6-15 days after birth) and mature milk (after 15 days) (Pons *et al.*, 2000). Colostrum is a richer source of nutrients and immune factors than mature milk, being the most potent natural immune booster known to science (Uruakpa *et al.*, 2002). It contains the highest concentration of proteins, mainly immunoglobulins (Igs) and lactoferrin, but also lysozyme and lactoperoxidase (LPO), which all have antimicrobial activity. These "bioactive proteins" are relatively resistant against proteolysis in the gastrointestinal tract and, thus, contribute to the defence of breastfed infants against pathogenic bacteria and viruses (Lönnerdal, 2003).

There are several classes of immunoglobulins (IgA, IgM, IgG, IgE and IgD) and IgA, IgM and IgG are the major ones present in human milk (Hurley & Theil, 2011). IgA is present in human milk in its secretory form (sIgA) – a dimer of IgA linked together with a secretory component and a joining chain – and represents the predominant immunoglobulin fraction (>90%) (Goldman, 1993). These specific Igs function by directly binding to specific microbial antigens, blocking binding and adhesion, enhancing phagocytosis, modulating local immune function, and contributing to the infant's immune system development (Lawrence & Pane, 2007).

Lysozyme (EC 3.2.1.17), on the other hand, acts by degrading the outer cell wall of Gram-positive bacteria by hydrolysing β-1,4 linkages between N-acetylmuramic acid and 2-acetylamino-2-deoxy-D-glucose residues (Chipman & Sharon, 1969). Besides causing bacterial cell wall lysis, lysozyme binds endotoxin (limiting its effect), increases IgA production, and contributes to macrophage activation (immunomodulatory effects) (Lawrence & Pane, 2007). Lactoperoxidase (EC 1.11.1.7; LPO), in the presence of hydrogen peroxide (H_2O_2; produced in small quantities by cells), catalyses the oxidation of thiocyanate (part of saliva) to antimicrobial compounds, such as hypothiocynate or higher oxyacids, which are able to kill both Gram-positive and Gram-negative bacteria (Lönnerdal, 2003).

Even though mother's own milk is the gold standard for infant nutrition, sometimes the mother cannot adequately supply her child. In this case donor human milk is a better alternative than bovine infant formulas, because donor milk has this great array of "bioactive factors" that cannot be mimicked by infant formula and make human breast milk so unique (Hamosh, 2001). However, because of the manner in which donor milk is collected, processed and stored in human

milk banks (HMB) these prized properties ascribed to mother's own milk are substantially diminished. The major process responsible for these losses is the low-temperature long-time thermal pasteurisation (LTLT), also known as Holder pasteurisation (62.5 °C for 30 min), which decreases IgA (up to 50%), IgG (34%) and lysozyme (up to 65%) contents, among others (Evans *et al.*, 1978; Heiman & Schanler, 2007), and destroys IgM in human milk (Ford *et al.*, 1977). Nevertheless, pasteurisation is a mandatory step to assure microbial safety of human milk. Hence, alternative pasteurisation technologies able to render better retention of human milk properties would be of great value.

High-pressure processing (HPP) is a novel non-thermal or cold (under refrigeration temperature) pasteurisation technology that is being increasingly applied in food industries worldwide, primarily as an alternative to thermal treatment, due to its capacity of providing safe foods with similar characteristics to the raw unprocessed foods (fresher-tasting and with better nutritive and functional properties). A prime example of HPP potential is the pressurised antibody-rich bovine colostrum beverage currently commercialised, that could not be accomplished by thermal pasteurisation (see Chapter 1). There are only few studies concerning the effect of HPP on mature human milk, which report 100% IgA retention after HPP at 400 MPa for 5 min at 12 ºC (Permanyer *et al.*, 2010) and IgA and lysozyme activities of 85.6% and 106.9%, respectively, after a 400 MPa pressure treatment for 30 min at 21 ºC (Viazis *et al.*, 2007).

The aim of this study was to investigate and compare the effects of HPP (200, 400 and 600 MPa for 2.5, 15 and 30 min at 8 ºC) and of a Holder-like thermal pasteurisation on total IgA, IgM and IgG contents, and lysozyme and lactoperoxidase activities in human colostrum. To our knowledge, this is the first work that evaluates HPP effects on human colostrum.

2. Materials and methods

2.1. Sample collection

Human colostrum samples from 11 healthy mothers attending Infante D. Pedro Hospital (Aveiro, Portugal) were collected into sterile colostrum containers, by manual or mechanical expression of the breast, using an electric breast pump for the mechanical expression. All the material was provided by Medela (Medela, Portugal). The samples were collected 1-4 days after term delivery and always in the morning after the first feed of the day, comprising a total volume of 35.2 mL. Exclusion criteria for the mothers were those followed by the Human Milk Banking Association of North America (HMBANA), except for the one that excludes women which have been in the United Kingdom for more than 3 months or in Europe for more than 5 years since 1980 (Human Milk Banking Association of North America). The study was previously approved by the Ethical Committee of Infante D. Pedro Hospital and informed consent was obtained from all the donor mothers (see Appendix A).

After collection, colostrum was transported to the laboratory in ice in less than 30 min, immediately frozen at -80 ºC and stored at -20 ºC until thermal or pressure treatments were performed. On the day of the treatments, the samples were thawed in a water bath, pooled to make a homogeneous batch and then divided into several aliquots for eleven different groups: one group of raw untreated colostrum, one to be thermally pasteurised, and nine for the various HPP treatments (200, 400 and 600 MPa for 2.5, 15 and 30 min each). Along these treatments samples were kept in ice slurry and at the end frozen at -80 ºC and stored at -20 ºC for subsequent analysis. Samples were stored at -20 ºC up to one week for lysozyme and lactoperoxidase activity measurement and up to one month for immunoglobulin content analysis.

2.2. Thermal pasteurisation

The samples were thermally pasteurised by the Holder or LTLT pasteurisation method, according to the general HMBANA procedure (Updegrove, 2005). Nine polypropylene tubes containing aliquots of 400 µL each were placed into a water bath at 62.5 ºC with one of them containing bovine milk and a thermocouple (instead of human colostrum) to estimate the time that colostrum would take to reach 62.5 ºC. Bovine milk was used instead of human colostrum to avoid spending colostrum, which is collected in very small amounts. Once that temperature was reached, the samples were held in the water bath for 30 min. Following pasteurisation, the tubes were quickly cooled in an ice slurry.

2.3. HPP treatments

The pressure treatments were carried out using a hydrostatic press from Unipress Equipment, Model U33 (Warsaw, Poland), with a pressure vessel of 100 mL (35 mm diameter and 100 mm height), surrounded by an external jacket, connected to a thermostatic bath to control the temperature. The unit has a maximum working pressure of 700 MPa and a working temperature between -20 ºC and 100 ºC. The pressure-transmitting fluid was a mixture of propylene glycol and water (60:40).

Nine distinct treatments were performed by combining three pressure levels with three treatment times: 200 MPa for 2.5, 15 and 30 min; 400 MPa for 2.5, 15 and 30 min; and 600 MPa for 2.5, 15 and 30 min. The initial temperature of the pressure vessel was set to 8 ºC. Time to reach the pressure was of 0.75, 1.12 and 1.23 min, respectively, for 200, 400 and 600 MPa, resulting in compression rates of, respectively, 264, 360 and 426 MPa/min. For each pressure treatment, colostrum was previously aliquoted to eight polypropylene tubes (400 µL each), which were then inserted into a small flexible plastic bag that was vacuum-sealed, placed inside another plastic bag and sealed again, with caution not to leave air inside the two bags.

2.4. Immunoglobulin content analysis

Immunoglobulins A, M and G were measured in human colostrum using Human IgA, IgM and IgG ELISA kits (KOMA BIOTECH), respectively, according to the manufacturer's instructions. ELISA (or enzyme-linked immunosorbent assay) is an immunoassay technique involving the reaction of antigen and antibody *in vitro*, and these kits are based on the sandwich ELISA type, elucidated in Figure 6. In order to describe the general procedures of the kits, the letter X will be used as a generic designation for any of the three Igs. The kits contained all of the required reagents and material for IgX quantitation: a pre-coated 96 well ELISA microplate (with antigen-affinity purified goat anti-human IgX), plate sealers, detection antibody (horseradish peroxidase conjugated antigen-affinity purified goat anti-human IgX), standard protein (human reference serum), assay diluent (1% bovine serum albumin), color development reagents (tetramethylbenzidine and H_2O_2 solutions), stop solution (2 M H_2SO_4), and washing solution (phosphate buffered saline powder with 0.05 % Tween-20, pH 7.4).

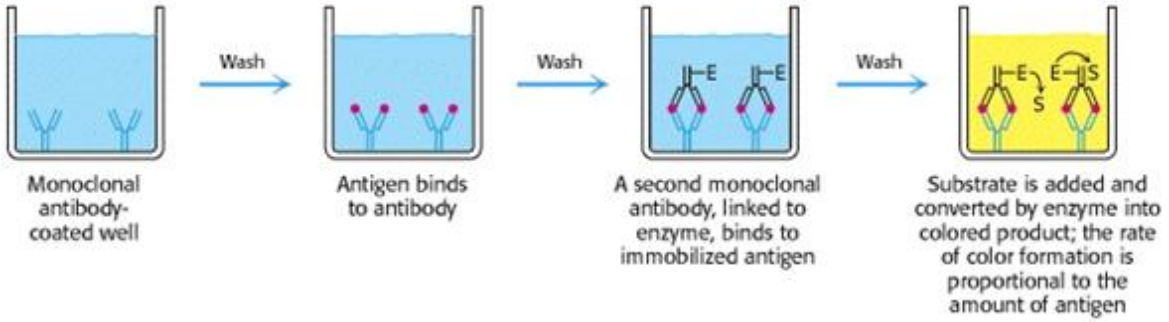

Figure 6. General steps of the sandwich ELISA assay.

In this assay, 100 µL of blank, standard or colostrum samples were added to each well of the pre-coated microplate in duplicate and incubated at room temperature for 1 h. The standards were diluted following the manufacturer's recommended dilutions and samples were diluted from 1:5000 to 1:10000, depending on the immunoglobulin. Then 100 µL of the diluted detection antibody (1:20000 to 1:50000) were added per well and incubated during 1 h at room temperature. Before, between and after the above steps the wells were aspirated to remove the liquid and washed five times with the washing solution to remove unbound molecules. Prior incubation the plates were covered with the plate sealers. For the color development reaction, 100 µL of color development solution were added to the wells and incubated for about 10 min for a proper color development. The reaction was stopped by adding 100 µL of the stop solution and the absorbance read at 450 nm in less than 20 min using a Multiskan GO microplate reader (Thermo). Total immunoglobulin concentrations were determined using the standard curves constructed with the diluted standards (Appendix B).

2.5. Lysozyme activity analysis

Lysozyme activity was determined using a *Micrococcus lysodeikticus* based turbidimetric procedure recommended by Sigma Chemical Co. (Sigma, St. Louis, MO), with minor modifications. The principle of this assay is the lytic activity of lysozyme towards *Micrococcus lysodeikticus* cell walls (antibacterial activity). A 0.015% (w/v) *Micrococcus lysodeikticus* cell suspension (substrate) was prepared by suspending *Micrococcus lysodeikticus* ATCC 4698 lyophilised cells in sodium phosphate buffer (66 mM, pH 6.24). To assure the freshness of this suspension, its absorbance at 450 nm (A_{450nm}) was measured at the beginning of the analyses and was consistently between 0.6 and 0.7, as it should. Prior to the enzymatic reaction, the substrate was heated at 30 ºC in a water bath. The reaction was initiated by adding 0.10 mL of appropriately diluted human colostrum to 2.50 mL of substrate and, after mixing by inversion, the decrease in A_{450nm} was immediately recorded for 3 min at 10 s intervals using a PerkinElmer Lambda 35 UV-

Vis spectrophotometer (PerkinElmer Instruments). Measurements were carried out against the reagent blank, containing the substrate and 0.10 mL of buffer, and each sample was measured in triplicate. The ΔA_{450nm}/min was obtained using the initial linear rate. One unit of lysozyme activity was defined as the amount of the enzyme that produces a ΔA_{450nm}/min of 0.001 per min at pH 6.24 at 30 ºC using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2.6 mL reaction mixture.

2.6. Lactoperoxidase activity analysis

LPO activity was determined according to the modified method of Pruitt and Kamau (1994). This method is based on the LPO catalysis of reactions in which H_2O_2 is reduced and a suitable electron donor is subsequently oxidised, such as reaction (1). 2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) was chosen, since it is accepted as the electron donor of choice for peroxidase assay. In its oxidised form ABTS absorbs light and can therefore be determined by a spectrophotometric assay.

$$ABTS_{reduced} + H_2O_2 \rightarrow 2\ ABTS_{oxidised} + 2\ H_2O \quad (1)$$

In this procedure, the substrates (ABTS and H_2O_2 solutions) were heated at 30 ºC in water bath and then 1 mL of 0.3 mM H_2O_2 was mixed with 2 mL of ABTS in phosphate buffer (0.1 mM, pH 6.7) in a cuvette. Reaction was initiated by adding 0.1 mL of human colostrum (diluted and undiluted) to the mixture and, after mixing by inversion, monitored for 12 h with the absorbance recorded at 412 nm (A_{412nm}). Measurements were carried out against the blank and each sample was measured in triplicate. One unit is defined as the amount of enzyme which oxidises 1 µmol ABTS/min at pH 6.7 at 30 ºC.

2.7. Statistical analysis

Differences between treatments were tested at a 0.05 level of significance. The effects of pressure level and holding time were tested in a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey's HSD) to find which treatments were significantly different from one another. All data are expressed as the mean ± standard deviation. The standard deviation was always ≤ 10%.

2.8. Kinetic data analysis

Denaturation kinetics of colostral Igs toward HPP was subjected to reaction kinetic analysis. Denauration of IgA, IgM and IgG at 400 and 600 MPa could be described by a first order model (2). According to Eq. (2), loss of Ig concentration rate ($-dA/dt$) is proportional to the denaturation rate constant (k) and the Ig concentration at each treatment time (A).

$$\frac{dA}{dt} = -kA \quad (2)$$

The reaction rate constant was determined from a semilogarithmic plot (3) of the Ig retention (A/A_0) as a function of the exposure time (t). A represents the response value after HPP treatment and A_0 is the initial value. D-values (decimal reduction time) were also calculated according to (4).

$$\ln\left(\frac{A}{A_0}\right) = -kt \quad (3)$$

$$D = \frac{\ln(10)}{k} \quad (4)$$

3. Results and discussion

3.1. Immnunoglobulins, lysozyme and lactoperoxidase in raw colostrum

Human colostrum contains a range of antimicrobial factors, including immunoglobulins, the antibacterial and lytic enzyme lysozyme and the antibacterial enzyme lactoperoxidase. The concentrations obtained for these factors in this work are reported in Table 8. The profile of immunoglobulins in human colostrum is similar to that found in mature milk, with IgA level high in both, particularly in colostrum. IgM is the second major class in human colostrum and, as well as IgA, is present in its secretory form (sIgM). Human colostrum has a low content of IgG, and the IgG required to provide systemic immunity is transferred across placenta before birth (Hurley & Theil, 2011). Lysozyme is also present in high amounts in human colostrum, but rather than slowly declining as lactation proceeds, lysozyme activity increases progressively (Goldman *et al.*, 1982; Prentice *et al.*, 1984). Human milk contains nearly 3000-fold lysozyme activity than bovine milk. Lactoperoxidase activity, on the other hand, is 100-fold higher in bovine milk than in human milk (Shahani *et al.*, 1980).

Table 8. Total IgA, IgM and IgG concentrations, and lysozyme and lactoperoxidase activity in raw colostrum samples.

Protein	Raw colostrum samples ± SD
Immunoglobulin A (mg/mL)	1.728 ± 0.034
Immunoglobulin M (mg/mL)	0.200 ± 0.011
Immunoglobulin G (mg/mL)	0.199 ± 0.010
Lysozyme (U/mL)	18033 ± 570
Lactoperoxidase (U/mL)	Not detected

In this work, IgA levels obtained for the untreated raw colostrum (Table 8) are in agreement with those of other authors (Shi *et al.*, 2011). Ronayne de Ferrer *et al.* (1984) found a slightly higher IgA concentration of 2.128 mg/mL in human milk 1-10 days postpartum (colostrum and transitional milk), but even higher IgA concentrations in colostrum (≈ 8-9 mg/mL) were found by other authors (Ramírez-Santana *et al.*, 2012; Yuen *et al.*, 2012). IgM concentration was slightly higher than that found in another study (0.12 mg/mL) (Shi *et al.*, 2011), and lower than that reported by Mickleson and Moriarty (1982) (mean of 0.58 mg/mL on day 3). IgG content is in agreement with that reported by other authors (mean of 0.19 mg/mL on day 3) (Mickleson & Moriarty, 1982). However, Shi *et al.* (2011) obtained a higher IgG concentration of 0.46 mg/mL.

Lysozyme and lactoperoxidase are two of the most studied enzymes in human milk, due to their antibacterial activity. Untreated colostrum samples had a mean lysozyme activity of 18033

U/mL, which, assuming the average protein content of mature human milk (≈ 8-10 g/L) (Lönnerdal, 2003; Zivkovic *et al.*, 2011), is very similar to that reported for mature milk (Viazis *et al.*, 2007). This assumption has to be made, since the authors reported the results per g of protein. Regarding lactoperoxidase, no activity was detected in raw colostrum in quantifiable levels. This is in accordance with a previous study (Ford *et al.*, 1977).

3.2. Effects of thermal pasteurisation and HPP on immunoglobulins

Several studies have already investigated the effect of thermal pasteurisation on human milk, but there are only a few investigating HPP effects on mature human milk, and this is the first one to assess high pressure effects on human colostrum, as the authors are aware. Currently, the Holder pasteurisation technique (62.5 ºC for 30 min) is the most commonly employed pasteurisation procedure in human milk banks of several hospitals. Thus, we performed a Holder-like pasteurisation in order to compare its effects with those of HPP on the selected bioactive components. Figure 7 presents the percentage of IgA, IgM and IgG retention in human colostrum after Holder pasteurisation and the various pressure treatments (200, 400 and 600 MPa for 2.5, 15 and 30 min at 8 ºC). IgA, IgM and IgG concentrations (mg/mL) before and after all of the treatments can be seen in Appendix C.

It is noteworthy that, due to the limited amount of sample and the variety of treatments to be performed, small aliquots (400 µL) were thermally pasteurised. As heat is transferred from the outside (water bath) to the inside of colostrum samples, forming a temperature gradient, an increase in the volume of colostrum to be treated would require more time to reach the temperature of 62.5 ºC in the "cold point", thus leading to more over-heated areas on the surface. As larger amounts of human milk are pasteurised in human milk banks, a greater destruction of its bioactive properties would be expected after Holder pasteurisation. High pressure is uniformly and nearly instantaneously transmitted throughout the food (Torres & Velazquez, 2008), so that its effects are independent of the volume of colostrum to be processed.

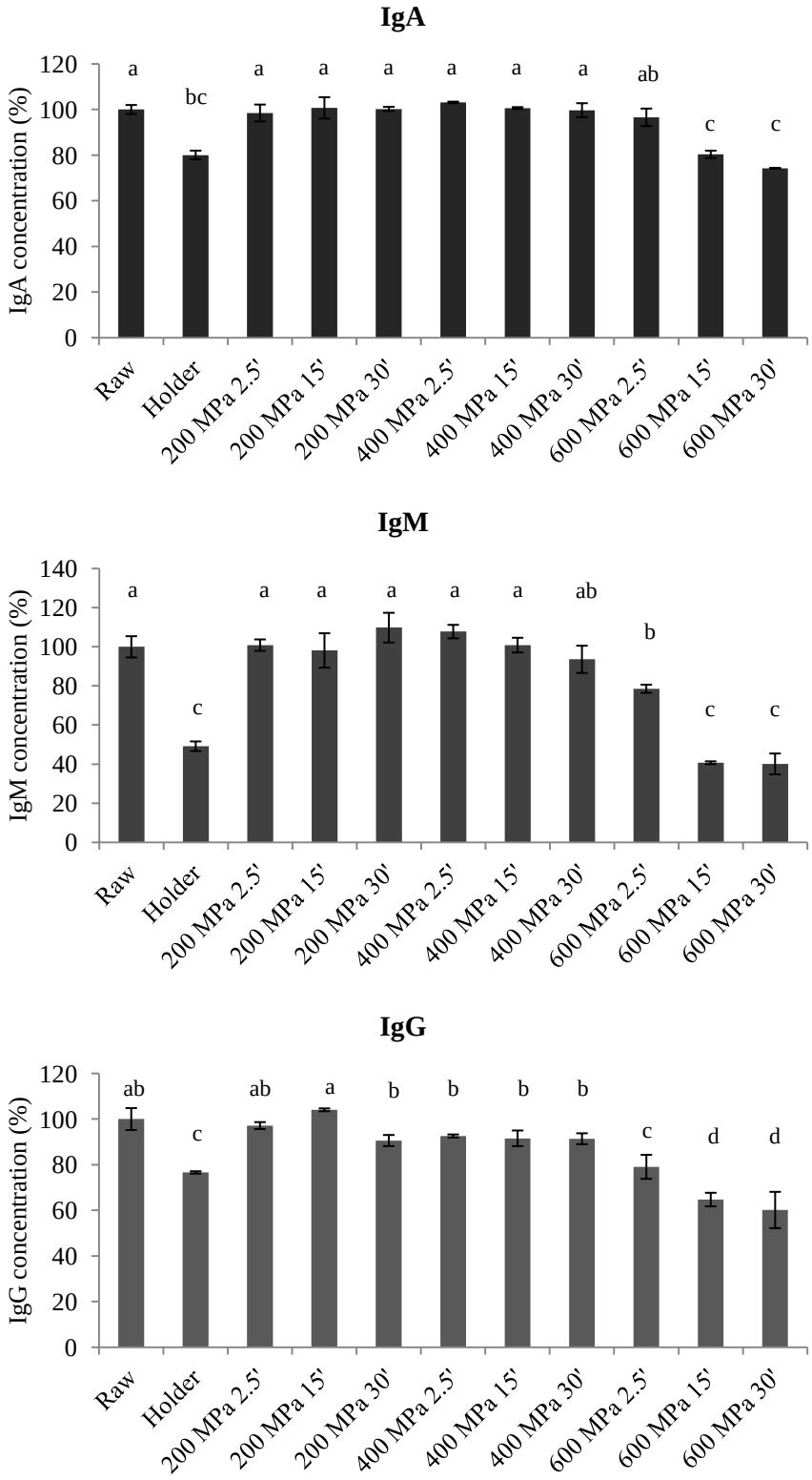

Figure 7. Percentage of IgA, IgM and IgG concentration in human colostrum before and after Holder pasteurisation and the HPP treatments studied. Data are presented as a percentage considering 100% the

value obtained for colostrum before any treatment (raw or unprocessed colostrum). Bars with the same letter are not statistically different ($p<0.05$).

3.2.1. IgA

Total IgA and sIgA are among the most studied human milk constituents (mainly in mature human milk), and diverse authors reported variable losses of this immune factor after Holder pasteurisation. Ford *et al.* (1977) found an IgA reduction of 20%, and, more recently, Permanyer *et al.* (2010) obtained a similar decrease (of 28%) in the IgA content. Also Czank *et al.* (2009) reached a sIgA decrease of 28% after LTLT pasteurisation of human milk. Accordingly, in the present study the LTLT Holder pasteurisation caused a significant decrease of 20% in human colostral total IgA ($p<0.05$; 80% retention). There are, however, studies that present higher IgA losses of 62% in colostrum (Koenig *et al.*, 2005) and of 64% mature human milk (Braga & Palhares, 2007; Viazis *et al.*, 2007), whereas other authors report no losses (Evans *et al.*, 1978).

Regarding the pressure treatments performed, our data show that IgA content is retained after HPP at 200 and 400 MPa for 2.5, 15 and 30 min, and 600 MPa for 2.5 min at 8 ºC (Figure 7, A). Increasing the hold time at 600 MPa for 15 and 30 min resulted in significant IgA losses of 20 and 26%, respectively, which are similar to those caused by Holder pasteurisation. These results are in agreement with those obtained by Permanyer *et al.* (2010) for mature human milk, as 100% IgA was maintained after HPP at 400 MPa and significant losses were verified after 600 MPa for a time higher than 2.5 min (5 min) at 12 ºC. Viazis *et al.* (2007), on the other hand, found significant reductions of 17 and 14% in IgA content and activity, respectively, after 400 MPa for 30 min at 21 ºC (estimated final temperature of 31 ºC). Therefore, it is arguable that the higher temperature at which human milk samples are pressure-processed can play a role in IgA partial inactivation. Either way, in all of the studies HPP at 400 MPa for various holding times proved to be more efficient than Holder pasteurisation in preserving the IgA content of human milk.

3.2.2. IgM and IgG

Although the effect of Holder pasteurisation on human milk IgM and IgG has not been as thoroughly studied as its effect on IgA, there is evidence that these Igs are not stable to such heat treatment (Ewaschuk, Unger, Harvey, *et al.*, 2011). IgM was found to be destroyed in human colostrum (Koenig *et al.*, 2005) and mature milk (Ford *et al.*, 1977) after Holder pasteurisation. In the last two studies IgM initial content was very low (0.014 and 0.10 mg/mL, respectively). In this work, IgM content of human colostrum was significantly reduced by 51% after thermal treatment ($p<0.05$). IgM content of unprocessed colostrum in the present work is twice as much as that reported by Ford *et al.* (1977) for raw human milk, and they heated higher quantities of milk (1.5 mL) than colostrum in the present work (400 µL), leading to more time to reach the desired

pasteurisation temperature (62.5 ºC). Therefore, it is likely that these two factors resulted in more IgM destruction.

Actually, there is still no literature concerning HPP effects on IgM or IgG contents in human milk. The present study shows no significant reduction of IgM in colostrum for a wide range of pressures and holding times (200 and 400 MPa for all studied holding times; $p>0.05$). At 600 MPa for 2.5 min IgM content is significantly reduced in 21%. The pressure treatments at 600 MPa for 15 and 30 min caused a significant destruction of IgM (59 and 60%, respectively), similar to that caused by the Holder-like pasteurisation (Figure 7).

The reports of IgG reduction caused by Holder pasteurisation in the literature are not consistent. While Evans *et al.* (1978) obtained 34% reduction of IgG content after pasteurisation of mature milk, Koenig *et al.* (2005) found more than twice as much IgG destruction (≈ 72%) in colostrum. Our results show a significant 23% colostral IgG reduction after Holder pasteurisation ($p<0.05$). In relation to the pressure treatments, HPP at 200 and 400 MPa preserves IgG content, as no significant differences were found after these HPP treatments ($p>0.05$; Figure 7). HPP at 600 MPa for 2.5 min resulted in a significant 21% decrease in IgG content (identical to the 23% loss after pasteurisation). The pressure treatments at 600 MPa for 15 and 30 min achieved the highest IgG reductions of 35 and 40%, respectively. These results confirm that HPP between 200 and 400 MPa up to 30 min maintains not only the IgA content in human milk (colostrum and mature milk), but also IgM and IgG concentrations in human colostrum.

3.3. Effects of thermal pasteurisation and HPP on lysozyme

Lysozyme is known to be highly heat stable at acidic pH, but heat labile at neutral pH. As human milk pH is around 7-7.4 (Morriss *et al.*, 1986), this enzyme is thus heat labile in human milk (Ford *et al.*, 1977). Significant losses in lysozyme content after Holder pasteurisation have been reported for human colostrum (≈ 74%) (Koenig *et al.*, 2005) and human milk (from 24 to 61%) (Czank *et al.*, 2009; Evans *et al.*, 1978). Moreover, Viazis *et al.* (2007) reported a 21% loss of lysozyme activity in mature human milk after Holder pasteurisation. The percentage of lysozyme activity retention found in this work for human colostrum after Holder pasteurisation and the various pressure treatments (200, 400 and 600 MPa for 2.5, 15 and 30 min at 8 ºC) is presented in Figure 8 (see Appendix C for lysozyme activity values (U/mL)). In accordance with the reported results, data of the present study show a significant decrease in lysozyme activity of 44% after pasteurisation of human colostrum ($p<0.05$). This decrease is, however, greater than that reported by Viazis *et al.* (2007).

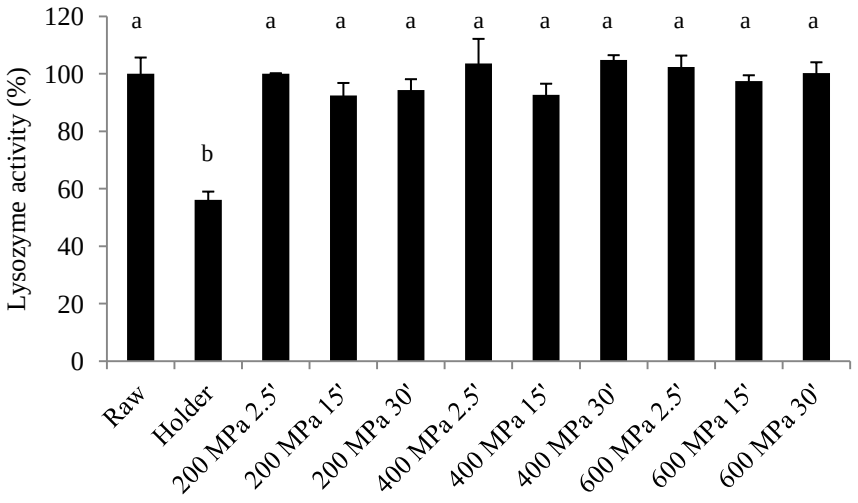

Figure 8. Percentage of lysozyme activity in human colostrum before and after Holder pasteurisation and the HPP treatments studied. Data are presented as a percentage considering 100% the value obtained for raw colostrum. Bars with the same letter are not statistically different ($p<0.05$).

All the pressure treatments studied did not cause significant differences in lysozyme activity ($p>0.05$) comparing to the raw unprocessed colostrum samples (Figure 8). Lysozyme activity was maintained after all of the HPP treatments, even at higher pressures and holding times. These results are in agreement with those presented by Viazis *et al.* (2007) for human mature milk. This pattern of enzyme activity retention after HPP has also been demonstrated for various enzymes in bovine milk. As reviewed by Huppertz *et al.* (2006), most milk enzymes are quite baroresistant, with plasmin, alkaline phosphatase, lactoperoxidase, xantine oxidase, phosphohexoseisomerase, γ-glutamyltransferase or lipase being resistant to pressures as high as 400 MPa. Besides, unlike thermal denaturation, pressure induced denaturation from pressures of 100 to 400 MPa may be reversible, probably because covalent bonds remain unaffected, making the effect of pressure only temporary (Hayakawa *et al.*, 1996).

3.4. Effects of thermal pasteurisation and HPP on lactoperoxidase

LPO activity was not detected in quantifiable levels neither in raw nor in any of the treated colostrum samples, even when longer reaction times were studied (up to 12 h) and higher amounts of colostrum were used. To confirm that the assay was working properly, tests were made using diluted bovine milk instead of human colostrum and 1 min was enough to record LPO activity, as reported by other authors (Fonteh *et al.*, 2002). As above referred, this is in accordance with a previous study (Ford *et al.*, 1977). Nevertheless, other authors were able to detect LPO activity in colostrum and mature milk using different substrates in the enzymatic assay (Gothefors &

Marklund, 1975; Shin *et al.*, 2000). Thus, to choose other assays with a lower detection limit may be an appropriate approach to quantify LPO activity in the future.

3.5. HPP denaturation kinetics of human colostrum immunoglobulins

As slight decreases in Igs concentration were detected after HPP of human colostrum at 400 MPa and significant losses were found after 600 MPa with increasing treatment times (0, 2.5, 15 and 30 min), these data were subjected to reaction kinetic analysis and could be described by a first order kinetic model (see Appendix D). The degree of Igs denaturation increased with pressure and treatment time. Data of Igs concentration at 200 MPa could not be adjusted to this model, as no linear decrease of Igs retention (A/A_0) with increasing treatment times was observed (Appendix D). The denaturation kinetic parameters *k* (reaction rate constant) and D (decimal reduction time) were calculated for HPP at 400 and 600 MPa and are presented in Table 9. It should be noted that, due to the limited treatment times studied, this is just a preliminary determination of these parameters. To obtain accurate *k* and D-values more treatment times have to be studied for each of these pressure levels. In any case, these preliminary data enable the attainment of some conclusions.

Table 9. Kinetic parameters for HPP denaturation of IgA, IgM and IgG in human colostrum, assuming a first order reaction.

Immunoglobulin	Pressure (MPa)	*k*-value (min^{-1})	r^2	D-value (min)
IgA	400	1.22×10^{-3}	0.917	1919
	600	1.46×10^{-2}	0.999	158
IgM	400	5.13×10^{-3}	0.999	452
	600	5.75×10^{-2}	0.989	40
IgG	400	4.66×10^{-4}	0.821	4941
	600	9.78×10^{-3}	0.905	235

Linear regression analysis showed high coefficients of correlation (except for IgG at 400 MPa), among those reported in literature for this type of analysis (Ludikhuyze *et al.*, 2001; Tayefi-Nasrabadi *et al.*, 2011). D-values for 400 MPa were almost 10-fold higher for IgA and IgM at 400 MPa than at 600 MPa, which means that treatments at 400 MPa require almost 10× more time to reduce these Igs concentration in one logarithmic cycle than treatments at 600 MPa, that is, to reduce its concentration in 90%. For IgG this difference is even more extended; D-value at 400 is about 20-fold higher than 600 MPa. When comparing the different Igs, it can be concluded that IgM is the most pressure labile (lowest D-values) and IgG the most pressure stable (highest D-values). This tendency of pressure stability is in agreement with literature regarding thermal stability of the same Igs (Ewaschuk *et al.*, 2011). Both pressure and thermal stability of IgA and

IgG are quite similar and those of IgM are much lower. Still, a study of thermal denaturation of buffalo milk Igs reported IgA as being the most thermal labile Ig, followed by IgM (El-Loly *et al.*, 2007).

To our knowledge, there are no reported data of high pressure denaturation of Igs in human colostrum or milk. For bovine milk IgG, the reported D-value at 600 MPa is lower than that obtained for human colostrum, about 71 min (Mazri *et al.*, 2012). As referred above, these are only preliminary results. The overall results of IgA, IgM and IgG seem to indicate that pressure denaturation at 600 MPa may follow a biphasic denaturation pattern, faster in the first 15 minutes and slower in the minutes after. These results give valuable preliminary information and should be considered in the design of pressure treatments of human colostrum to preserve its Igs structure and thus biological function.

4. Conclusions

In the present work, it was found that HPP of human colostrum at pressures of 200 and 400 MPa for 2.5, 15 and 30 min did not significantly decrease IgA, IgM and IgG contents. For IgA, HPP at 600 MPa for 2.5 min did not cause significant differences as well, when comparing to raw colostrum concentrations. In contrast, Holder pasteurisation significantly reduced IgA, IgM and IgG initial concentration in 20, 51 and 23%, respectively. Pressure processing at 600 MPa for 15 and 30 min also caused significant reductions in all of the Igs, similar or even greater than those caused by Holder pasteurisation. It was verified that IgM is the most pressure labile Ig (lower D-value) and IgG the most pressure stable (higher D-value). Regarding lysozyme activity in human colostrum, the wide range of pressure treatments conducted was able to maintain this antimicrobial enzyme activity. Holder pasteurisation, on the other hand, caused a significant decrease of 44% in its activity.

We may conclude that HPP at 200 and 400 MPa (for all of the holding times tested) permits a much better retention of IgA, IgM, IgG and lysozyme in human colostrum than Holder pasteurisation, but HPP at 600 MPa for 15 and 30 min does not. Therefore, HPP is a potential alternative for the pasteurisation of this valuable food for newborns. Nevertheless, further research is required to determine HPP capacity of retaining other nutritional and functional components of human colostrum in particular, and human milk in general.

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CHAPTER 3

Effect of Thermal Pasteurisation and High-Pressure Processing on Immunoglobulin Content and Antimicrobial Enzyme Activity in Human Milk

Abstract

Immunoglobulins (Igs) A, M and G, lysozyme and lactoperoxidase in human milk play an important role in the prevention of infectious diseases, and are not present in bovine-based infant formulas. They are, however, strongly decreased after Holder pasteurisation in human milk banks. The aim of this work was to assess high-pressure processing (HPP) as a potential alternative to Holder pasteurisation. Mature human milk was subjected to various HPP treatments and Holder pasteurisation, and examined for its content of Igs, lysozyme and lactoperoxidase. HPP at 200 and 400 MPa at 8 ºC proved to retain much higher contents of IgA, IgM and IgG than pasteurisation, which decreased them in 27, 69 and 26%, respectively. Pressure treatments at 600 MPa for 2.5 min were also more effective than pasteurisation in retaining these biochemical components, but 600 MPa for 15 and 30 min is no longer a better alternative. Lysozyme activity was maintained or even increased after all of the HPP treatments at 8 ºC, and 50% inactivated by Holder pasteurisation. Furthermore, HPP at higher temperatures (40 ºC) significantly increased lysozyme activity in 43, 50 and 16% for 500, 700 and 900 MPa, respectively, during 15 min. The milk contained no detectable lactoperoxidase activity.

1. Introduction

The World Health Organization (WHO), the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN), and many professional associations and health care workers recommend exclusive breastfeeding for the first 6 months of life and continuation of breastfeeding after the introduction of complementary foods up to two years and beyond, as long as mutually desired by mother and child (Agostoni *et al.*, 2009; World Health Organization, 2010). Moreover, these organizations strongly advise and encourage legal regulations that facilitate breastfeeding, such as allowance of maternity leave for at least 6 months and protection of working mothers. There are several indicators of breast milk benefits in infancy and even later in the child's life; from reducing the risk of infectious diseases like necrotising enterocolitis (NEC), diarrhoea, respiratory tract infections and acute otitis media (Boyd *et al.*, 2007; Quigley *et al.*, 2007; Sabirov *et al.*, 2009) to helping prevent obesity (Arenz *et al.*, 2004) and death. It has been reported that about 1.45 million children's lives are lost per year due to suboptimal breastfeeding in developing countries (Lauer *et al.*, 2006).

There are numerous immunomodulatory components in breast milk, which are responsible for its effects in preventing or reducing the above referred complications. For instance, immunoglobulin (Ig) transfer through milk is key in providing passive local immunity to the newborn, mostly IgA and, in a lesser extent, IgM and IgG (Hurley & Theil, 2011). Besides, there are two major enzymes with antimicrobial activity in human milk that also may impact the infant's immune system: lysozyme and lactoperoxidase. These components are particularly invaluable to sick, low-birth-weight and extremely low-birth-weight infants who are born with a very naive immune system and are often hospitalised for an extended period in a neonatal intensive care unit (NICU). When mother's breast milk is unavailable, donor human milk is preferred to cow's milk-based infant formula, as it is not only much closer to mother's own milk in its overall nutritional composition (e.g. lower energy and protein contents), but also avoids exposure of the infant's gut to xenogeneic proteins and provides all of its natural nutritive and non-nutritive bioactive components that infant formulas cannot supply (Akinbi *et al.*, 2010; Hamosh, 1997).

Donor milk is commonly pasteurised in human milk banks (HMB) to avoid potential transmission of infectious agents, typically by heating to 62.5 ºC for 30 min (Holder pasteurisation) (Updegrove, 2005), a procedure that greatly decreases bioactive and immunoactive components of human milk. This subject has been recently reviewed, and IgA, IgM, IgG and lysozyme levels can be reduced up to 48, 100, 34 and 60%, respectively (Ewaschuk *et al.*, 2011). These losses of immune factors through pasteurisation may have significant clinical implications to infants at high risk of developing infectious diseases, such as NEC. Therefore, alternative pasteurisation technologies able to preserve immune components while inactivating potential pathogens should be

undoubtedly explored. High-pressure processing (HPP) technology has already proven to be able to answer this challenge for various foods.

HPP effects on foods were first studied more than a century ago, when it was observed that processing milk at 670 MPa for 10 min resulted in five to six microbial logarithmic reductions (Hite, 1899). Furthermore, high pressures are uniformly and nearly instantaneously transmitted throughout the foods, and they do not affect covalent bonds, only weaker bonds and forces (e.g. hydrogen bridges, electrostatic interactions and van der Walls forces). These features ascribe HPP many advantages over thermal and other non-thermal processes, namely short processing times, minimal heat exposure, no over-processed areas, and retention of sensory properties, nutrients and bioactive compounds (Schaschke, 2011). Even though this technology took a long time to reach the market (in 1980s), in just two decades HPP has clearly stood out from the other emergent preservation technologies, owing to its capacity of fully satisfying the need for fresh, nutritive, long shelf life products (Patterson *et al.*, 2006). However, HPP technology alone is limited to pasteurisation treatments. Sterilization of foods is only possible by combining high pressures with high temperatures in pressure-assisted thermal sterilization (PATS) (Mújica-Paz *et al.*, 2011). Pressure treatments at temperatures higher than 50 ºC are reported to be effective for some bacterial spores (Paredes-Sabja *et al.*, 2007). So far, only few studies have been conducted with respect to HPP effect on human milk components (Molto-Puigmarti *et al.*, 2011; Permanyer *et al.*, 2010; Viazis *et al.*, 2007).

The objectives of the present study were to investigate HPP effects on IgA, IgM, IgG and the antibacterial enzymes lysozyme and lactoperoxidase, and to compare them with those of Holder pasteurisation. Pressure treatments of 200, 400 and 600 MPa for 2.5, 15 and 30 min at 8 ºC were carried out, in order to assess HPP effects at various pressure levels and holding times, thus, allowing an overview of HPP performance regarding these human milk proteins. Additionally, the effect of HPP at particularly high pressures combined with high temperatures (500, 700 and 900 MPa at 40, 60 and 80 ºC for 15 min) in lysozyme activity was determined. This is the first study to determine the combined effect of temperature and pressure in human milk.

2. Material and methods

The methods used in this chapter for immunoglobulin and enzyme activity analysis were the same as those used in Chapter 2 with some minor modifications; thereby in this chapter they will just be briefly explained.

2.1. Human milk samples

Human milk samples were obtained from 7 healthy volunteer mothers 20-90 days after term delivery (mature human milk) at Infante D. Pedro Hospital in Aveiro, comprising a total volume of 150 mL. All the mothers gave their informed consent to participate in this study after receiving information about the aim and required procedures (Appendix A). The study was previously approved by the Hospital Ethical Committee. Exclusion criteria for the mothers were the following: a positive blood test result for HIV, HTLV, hepatitis B or C, or syphilis; use of illegal drugs, tobacco products, smoking, or excessive alcohol consumption; malnutrition; and recent infectious diseases. Human milk was collected into sterile polypropylene containers, by manual or mechanical expression of the breast, using a Swing™ electric breast pump for the mechanical expression provided by Medela (Medela, Portugal). After collection, human milk samples were immediately frozen in the hospital and then transported to the laboratory in ice and stored at -20 ºC. A few weeks later they were thawed in a water bath, pooled together, divided into aliquots and assigned to eleven different groups: one of untreated mature milk, one to be thermally pasteurised, and nine for the various HPP treatments (200, 400 and 600 MPa for 2.5, 15 and 30 min each). During the day of the treatments samples were kept in an ice bath and then frozen at -80 ºC and stored at -20 ºC up to two months until analysis.

Frozen human milk samples used for the pressure treatments at high temperatures (500, 700 and 900 MPa at 40, 60 and 80 ºC each) were provided by Instituto Tecnológico Agroalimentario de Extremadura (INTAEX, Badajoz, Spain) and all of these treatments were performed there. The samples were collected from Spanish mothers at 6 months postpartum. These samples were thawed on the day of the pressure treatments, kept in ice until processed, and frozen again at the end of the day at -80 ºC. The next day they were transported in ice boxes frozen to Aveiro, Portugal, and stored at -20 ºC for subsequent analysis. The analyses were performed in less than a month after freezing.

2.2. Thermal pasteurisation

Thermal pasteurisation was carried out by mimicking the Holder method (62.5 ºC for 30 min), according to the HMBANA procedure (Updegrove, 2005). Nine polypropylene tubes containing 1.5 mL of human milk each were placed in a water bath that was previously preheated

to 62.5 ºC. Another tube with the same amount of semi-skimmed cow's milk was placed in the centre of them with a thermocouple in the middle as a control of the time that human milk would need to reach the desired temperature of 62.5 ºC. Once that temperature was reached, the samples were held in the water bath for 30 min. After pasteurisation, the tubes were quickly cooled in an ice slurry and immediately frozen at -80 ºC.

2.3. High-pressure processing

The pressure treatments at 8 ºC were conducted using a hydrostatic press from Unipress Equipment, Model U33 (Warsaw, Poland), with a pressure vessel of 100 mL (35 mm diameter and 100 mm height), surrounded by an external jacket, connected to a thermostatic bath to control the temperature. The unit has a maximum working pressure of 700 MPa and a working temperature between -20 ºC and 100 ºC. The pressure-transmitting fluid was a mixture of propylene glycol and water (60:40). Nine distinct treatments were performed: 200 MPa for 2.5, 15 and 30 min; 400 MPa for 2.5, 15 and 30 min; and 600 MPa for 2.5, 15 and 30 min. Time to reach the pressure was of 0.75, 1.12 and 1.23 min for 200, 400 and 600 MPa, respectively, resulting in compression rates of 264, 360 and 426 MPa/min. Before each pressure treatment, human milk was aliquoted to nine polypropylene tubes (1.5 mL each), which were inserted into a small flexible plastic bag that was vacuum-sealed, placed inside another plastic bag and sealed again, with caution not to leave air inside the two bags.

For the pressure treatments combined with high temperatures, a thermostated multivessel high pressure equipment (Resato) was used. This equipment has a maximum working pressure and temperature of 1000 MPa and 100 ºC, respectively. Three different pressure levels were combined with three different temperatures: 500 MPa at 40, 60 and 80 ºC; 700 MPa at 40, 60 and 80 ºC; and 900 MPa at 40, 60 and 80 ºC for 15 min. For the treatments, 10 mL of human milk were pipetted in two small flexible plastic bags per treatment, which were vacuum-sealed and inserted into another plastic bag.

2.4. Immunoglobulin analysis

Total IgA, IgM and IgG were measured in human milk using Human IgA, IgM and IgG ELISA kits (KOMA BIOTECH), respectively, according to the manufacturer's instructions. In all of the procedures, 100 µL of blank, standard or human milk samples were added to each well of the pre-coated microplate and incubated at room temperature for 1 h with the plate sealer provided. The standards were diluted following the manufacturer's recommended dilutions and samples were diluted from 1:3000 to 1:5000, depending on the Ig. Then 100 µL of the diluted detection antibody (1:20000 to 1:50000) were added per well and incubated during 1 h at room temperature in the

sealed plate. Before, between and after the above steps the wells were aspirated to remove the liquid and washed five times with the washing solution to remove unbound molecules. For the color development reaction, 100 µL of color development solution were added to the wells and incubated for about 10 min for a proper color development. The reaction was stopped by adding 100 µL of the stop solution and the absorbance read at 450 nm in less than 20 min using a Multiskan GO microplate reader (Thermo Scientific). Duplicate determinations were performed for each Ig, and total immunoglobulin concentrations were determined using the standard curves constructed with the diluted standards (Appendix B).

2.5. Antimicrobial enzyme activity analysis

Lysozyme activity was determined using a *Micrococcus lysodeikticus* based turbidimetric assay recommended by Sigma Chemical Co. (Sigma, St. Louis, MO). Briefly, 2.50 mL of 0.015% (w/v) *Micrococcus lysodeikticus* cell suspension (substrate) at 30 ºC were mixed with 0.10 mL of diluted human milk and the absorbance at 450 nm (A_{450nm}) was immediately recorded for 3 min. Measurements were carried out against the bank and each sample was measured in triplicate. The ΔA_{450nm}/min was calculated from the slope of the linear part of the regression line when the absorbance decrease was plotted versus reaction time. One unit of lysozyme activity was defined as the amount of enzyme that produces a ΔA_{450nm}/min of 0.001 per min at pH 6.24 at 30 ºC using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2.6 mL reaction mixture.

LPO activity was measured spectrophotometrically at 412 nm and 30 ºC with 2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) in phosphate buffer (0.1 mM, pH 6.7) and 0.3 mM H_2O_2, according to the modified method of Pruitt and Kamau (1994). The reaction was monitored for 12 h. Each sample was analysed in triplicate and against the blank.

2.6. Statistical analysis

Differences in the concentration of IgA, IgM and IgG, and in the activity of lysozyme between treated and untreated samples and between different treatments were assessed at a 0.05 level of significance in a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey's HSD). The data are expressed as mean ± standard deviation, and the standard deviation was always < 15 %.

2.7. Kinetic data analysis

Isobaric denaturation of human milk IgM and IgG at 600 MPa could be described by a first order model (2), allowing the reaction rate constant (*k*) to be determined from a semilogarithmic plot (3) of the Ig retention (A/A_0) as a function of treatment time (*t*). D-values (decimal reduction time) were also calculated according to (4).

$$\frac{dA}{dt} = -kA \tag{2}$$

$$\ln\left(\frac{A}{A_0}\right) = -kt \tag{3}$$

$$D = \frac{\ln(10)}{k} \tag{4}$$

3. Results and discussion

3.1. Effects of thermal pasteurisation and HPP on immunoglobulins

As Holder pasteurisation of donor milk in HMB is a mandatory step to ensure microbial safety, but results in variable nutritional and non-nutritional losses, in the search for an alternative pasteurisation technology, HPP effects on human milk IgA, IgM and IgG contents for a wide range of pressures and holding times were determined. Holder pasteurisation was also tested with the aim of comparing the effects of both technologies. Table 10 presents IgA, IgM and IgG concentrations in raw and treated human milk. This data was also converted to percentages, considering 100 % the value obtained for raw human milk, and represented in Figure 9, in order to help establish comparisons between the distinct treatments effects.

Table 10. IgA, IgM and IgG concentrations and lysozyme activity in mature human milk before and after Holder pasteurisation and the various HPP treatments.

Treatment	IgA concentration (mg/mL)	IgM concentration (mg/mL)	IgG concentration (mg/mL)	Lysozyme activity (U/mL)
Raw	0.826 ± 0.066	0.178 ± 0.010	0.048 ± 0.002	22453 ± 141
Holder	0.606 ± 0.049	0.054 ± 0.001	0.035 ± 0.002	11180 ± 466
200 MPa 2.5’	0.837 ± 0.017	0.175 ± 0.006	0.046 ± 0.001	22933 ± 1546
200 MPa 15’	0.815 ± 0.033	0.174 ± 0.008	0.045 ± 0.003	24867 ± 599
200 MPa 30’	0.834 ± 0.051	0.174 ± 0.002	0.050 ± 0.002	21360 ± 682
400 MPa 2.5’	0.926 ± 0.059	0.169 ± 0.008	0.048 ± 0.002	21600 ± 1221
400 MPa 15’	0.828 ± 0.023	0.166 ± 0.004	0.055 ± 0.002	21500 ± 271
400 MPa 30’	0.817 ± 0.031	0.167 ± 0.003	0.046 ± 0.002	26573 ± 675
600 MPa 2.5’	0.945 ± 0.009	0.112 ± 0.001	0.048 ± 0.000	21247 ± 830
600 MPa 15’	0.588 ± 0.001	0.067 ± 0.005	0.035 ± 0.003	24720 ± 523
600 MPa 30’	0.567 ± 0.041	0.041 ± 0.000	0.033 ± 0.002	24053 ± 201

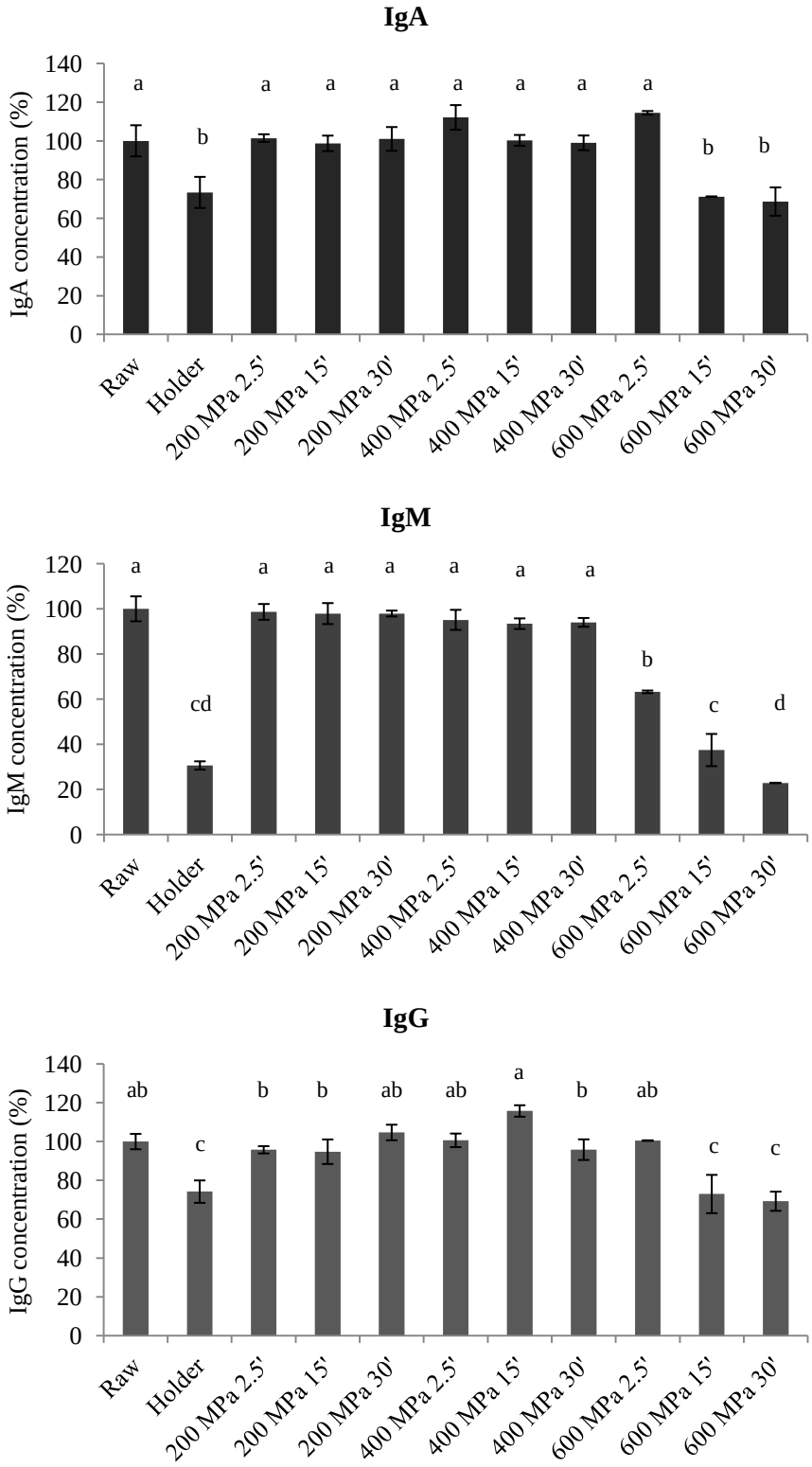

Figure 9. Percentage of IgA, IgM and IgG concentration in human milk before and after Holder pasteurisation and the various HPP treatments performed. Bars with the same letter are not statistically different ($p<0.05$).

3.1.1. IgA

Total IgA concentration obtained for raw human milk in this study (Table 10) is among those reported by various other authors (Mickleson & Moriarty, 1982; Viazis *et al.*, 2007; Weaver *et al.*, 1998), even though there are some variations. Ford *et al.* (1977) and Permanyer *et al.* (2010) found slightly lower IgA concentrations of 0.5, and 0.247-0.488 mg/mL, respectively. This is understandable, once human milk composition varies over the course of lactation and between lactating women (Prentice *et al.*, 1984), for example with the mother's dietary habits and nutritional status (Garg *et al.*, 1988). Holder pasteurisation caused a significant 27% decrease in human milk IgA content ($p<0.05$; Figure 9), which is consistent with the decreases found in other studies for mature milk (Czank *et al.*, 2009; Ford *et al.*, 1977; Permanyer *et al.*, 2010) and in Chapter 2 for colostrum.

IgA concentration in human milk was maintained after HPP treatments at 200 and 400 MPa for all holding times, and 600 MPa for 2.5 min (Figure 9). After 400 and 600 MPa for 2.5 min IgA content was increased in 12 and 14%, but these increases are not statistically significant ($p>0.05$). On the other hand, after 600 MPa for 15 and 30 min IgA retention was significantly decreased to 71 and 69%, respectively ($p<0.05$). This is probably due to changes in the secondary structure of IgA, which lead to protein denaturation. As reviewed by Hendrickx *et al.* (1998), hydrogen bonds are responsible for maintaining the helical structure of peptides, and are enhanced at low pressures and ruptured at very high pressures. Likewise, the rupture of ionic bonds is strongly affected by pressure increases. The same explanation applies to IgM or IgG in human milk.

The retention at 600 MPa for 15 and 30 min is similar to that of Holder pasteurised milk, and hence HPP treatments at 600 MPa for 15 min or more are no longer a better alternative to Holder pasteurisation in what concerns IgA retention. Also in the previous study with human colostrum (Chapter 2) this same behaviour was observed. In agreement with this, other authors reported 100% IgA maintenance after 400 MPa for 5 min at 12 ºC, and a decrease to 69% after HPP at 600 MPa for 5 min (Permanyer *et al.*, 2010). Similarly, in this work it was verified that IgA content is retained after HPP at 400 MPa regardless of treatment times, and reduced to 71% after pressure processing at 600 MPa for 15 min. Besides, when crossing these data, it can be inferred that pressure processing at 600 MPa for short times might cause no significant differences in IgA content (as observed for 2.5 min) and may be a possible alternative to thermal pasteurisation as well.

3.1.2. IgM and IgG

After IgA, which is the major immunoglobulin present in human milk, IgM and IgG are the second and third main Igs. In this work, IgM and IgG mean concentrations in raw human milk were

of 0.178 and 0.048 mg/mL, respectively (Table 10). IgM content is in accordance to that found by other authors for mature human milk (Ford *et al.*, 1977; Shi *et al.*, 2011), yet slightly higher, and is slightly lower than that determined for colostrum (Chapter 2). Nevertheless, this concentration is almost 4 and 6-fold higher than those determined by Mickleson and Moriarty (1982) for mature breast milk of women from New Zeland at 42 and 70 days postpartum, and similar to IgM concentration in milk at 14 days postpartum. IgG concentration, in its turn, is more similar to those found by these authors at 14, 42 and 70 days after delivery, and 4-fold lower than the mean IgG concentration found by Shi *et al.* (2011) for Chinese women. These last authors actually observed a higher concentration of IgG than IgM in human milk throughout lactation, which is uncommon. Some authors did not even detect IgG in human milk (Ford *et al.*, 1977).

After Holder pasteurisation IgM concentration was significantly reduced in 69% ($p<0.05$; Figure 9). Even though IgM concentration was greatly reduced after Holder pasteurisation, in other studies it was shown to be completely destroyed (Ford *et al.*, 1977; Koenig *et al.*, 2005). In the previous study with human colostrum (Chapter 2) a lower IgM reduction of about 51% was found. The several pressure treatments conducted resulted in less IgM losses than Holder pasteurisation (excepting for the one at 600 MPa for 30 min). HPP at 200 and 400 MPa for 2.5, 15 and 30 min did not cause significant reductions in IgM content ($p>0.05$; Figure 9). Nevertheless, after 600 MPa for 2.5 min IgM content was significantly reduced to 63%. Moreover, the pressure treatments at 600 MPa for 15 and 30 min led to significant IgM destruction of 63 and 77% (37 and 23% retention), respectively. These two last HPP treatments caused identical losses to Holder pasteurisation. When comparing to HPP effect on IgM of human colostrum (Chapter 2), these results are similar for 200 and 400 MPa and are somewhat different for HPP at 600 MPa, since colostral IgM was less reduced after 2.5, 15 and 30 min at 600 MPa.

Regarding IgG, its concentration was significantly decreased by 26% after Holder pasteurisation ($p<0.05$). IgG reduction of pasteurised milk in this work is in agreement with the 34% reduction obtained by Evans *et al.* (1978) and the 23% reduction obtained for colostrum (Chapter 2). In contrast to pasteurisation, IgG was not significantly reduced after any of the treatments at 200 and 400 MPa, and 600 MPa for 2.5 min ($p>0.05$; Figure 9). After 400 MPa for 15 min it was even increased in 16%, but this increase was not statistically significant. HPP at 600 MPa for 15 and 30 min caused significant IgG reductions, of 27 and 31% (73 and 69% retention), respectively, which are not statistically different from those caused by thermal pasteurisation. These results are consistent with those reported for human colostrum (Chapter 2), except for the HPP treatments of 400 MPa for 15 min, that did not cause a significant increase in IgG content, and 600 MPa for 2.5 min that led to a significant 21% IgG reduction. Overall, it was verified that also

for mature human milk HPP at 200 and 400 MPa up to 30 min maintains IgM and IgG much more effectively than the currently employed Holder pasteurisation.

3.2. Effects of thermal pasteurisation and HPP on antimicrobial enzyme activity

Lysozyme and lactoperoxidase are the two most noted antimicrobial enzymes in milk, even though lysozyme is present in human milk in higher quantities than lactoperoxidase (Shahani *et al.*, 1980). Lysozyme activity in raw and treated samples was analysed and is presented in Table 10. Lysozyme activity retention (%) was also plotted in Figure 10 to help the comparison of these results. Lactoperoxidase activity is not presented, as no activity was found in quantifiable levels in any of the treated and untreated human milk samples for 12 h. In agreement with this, Ford *et al.* (1977) also did not detect lactoperoxidase activity in mature milk. Thus, in the future other assay methods with lower sensitivity limits should be tested. Anyway, it may be concluded that this enzyme has a low activity in the human milk used. Lysozyme activity in raw milk determined in this work (Table 10) is slightly higher than that of human colostrum (Chapter 2), which agrees with the reported fact that lysozyme activity does not decrease along lactation, as many other proteins and antimicrobial factors; instead it increases progressively throughout lactation (Prentice *et al.*, 1984). This activity is also similar to that presented by Viazis *et al.* (2007), if one considers the average protein content of human milk reported in literature (≈ 8-10 g/L) (Lönnerdal, 2003).

It is well known that lysozyme content and activity decrease after thermal LTLT pasteurisation. Evans *et al.* (1978) found a 24% decrease in lysozyme content, while Czank *et al.* (2009) found a 61% destruction of lysozyme in mature human milk. In addition, Viazis *et al.* (2007) reported a 21% loss of activity after Holder pasteurisation. A much higher 50% loss of activity was obtained in this work, as represented in Figure 10. This significant loss ($p<0.05$) is consistent with that found in human colostrum after pasteurisation, of 44% (Chapter 2).

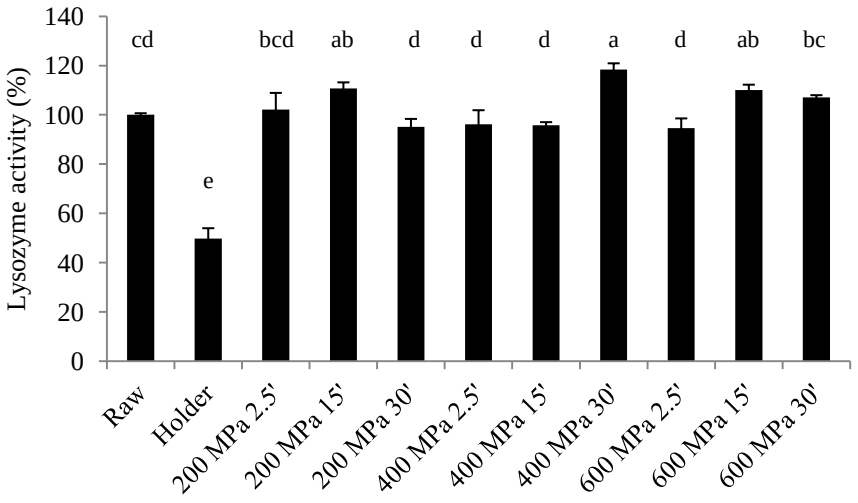

Figure 10. Percentage of lysozyme activity in human milk before and after Holder pasteurisation and the various HPP treatments performed. Bars with the same letter are not statistically different ($p<0.05$).

None of the pressure treatments caused significant reductions in lysozyme activity. On the contrary, pressure treatments at 200 MPa for 15 min, 400 MPa for 30 min and 600 MPa for 15 min led to significant 11, 18 and 10% increases in this enzyme activity, respectively ($p<0.05$; Figure 10). This lysozyme activity retention pattern after HPP was also found for colostrum (Chapter 2) and had already been demonstrated by Viazis *et al.* (2007) at 400 MPa up to 120 min. After 400 MPa for 30 min these authors also observed an increase in lysozyme activity (of 7%), even though it was not significant. Besides, Tedford *et al.* (1999) reported that bovine milk lysozyme was relatively pressure-resistant, with small structural modifications after HPP up to 600 MPa and 30 min. The increase in enzyme activity after HPP was reported by various authors for other enzymes as well, so a few examples will be presented. Pressure-induced activation of pectin methyl esterase (PME) in orange juice was achieved after HPP at 200-400 MPa at room temperature (Cano *et al.*, 1997). The activation of polyphenoloxidase activity in pears at 400-600 MPa at 20 ºC was also reported (Asaka *et al.*, 1994). Moreover, polygalacturonase activity was increased after diced tomatoes were pressurized at 400 MPa for 5 min at 25 ºC (Shook *et al.*, 2001), but the activity decreased increasing processing time and pressure.

In the present study, a maintenance or increase in lysozyme activity was observed for the wide range of pressures tested. The increase in its biological activity may be explained by the occurrence of conformational changes (Asaka *et al.*, 1994) or high pressure dissociation of lysozyme aggregates, making the enzyme more available of exerting its activity (Seefeldt *et al.*, 2009). These results are very interesting, once lysozyme maintenance in human milk may help protect the infants against bacterial infections and also contribute to microbial safety of human milk for increased periods.

3.3. HPP denaturation kinetics of human milk immunoglobulins

Unlike what was verified for human colostrum (Chapter 2), in human milk only HPP denaturation of IgM and IgG could be described by a first order kinetic model (Appendix E), after reaction kinetic analysis of all Igs. Regarding IgA, none of the pressure treatments allowed the establishment of a linear decreasing tendency in IgA retention (A/A_0) for the treatment times studied (more times should be studied in the first 15 min). For IgM and IgG, linear decreases were found just for the pressure treatment at 600 MPa. In these last cases, denaturation kinetic parameters *k* (reaction rate constant) and D (decimal reduction time) were calculated and are presented in Table 11. Besides, due to the limited treatment times studied, this is a preliminary determination of *k* and D-values. More treatment times have to be studied in order to further refine these values.

Table 11. Kinetic parameters for HPP denaturation of IgM and IgG in human milk, assuming a first order reaction.

Immunoglobulin	Pressure (MPa)	*k*-value (min^{-1})	r^2	D-value (min)
IgM	600	4.49×10^{-2}	0.936	51
IgG	600	1.32×10^{-2}	0.814	174

Coefficients of correlation (r^2) are not very high, but are among those reported in literature for this type of analysis (Ludikhuyze *et al.*, 2001). D-value of IgM is much lower (more than 3-fold) than that of IgG, showing the higher pressure sensitivity of IgM. This is in agreement to what was found for human colostrum (Chapter 2). In addition, IgM D-value at 600 MPa for human milk was similar to the same value for human colostrum (40 min). IgG D-value, in its turn, is lower than that found for human colostrum. This value is more close, yet superior, to that reported by Mazri *et al.* (2012) for bovine milk. As far as the authors are aware, this is the first study of high pressure denaturation of IgM and IgG in human milk, and there are not any in human colostrum, as well. Similarly to what was verified for human colostrum, it seems that denaturation may occur more rapidly in the first 15 min of pressure exposure and more slowly after that period of time (biphasic denaturation pattern). Nevertheless, more treatment times in the studied range (0-30 min) and in an extended range (e.g. up to 120 min) should be tested in order confirm these preliminary assumptions.

3.4. Effects of high pressure combined with high temperature on lysozyme activity

As it was observed that lysozyme was pressure-resistant to pressures up to 600 MPa for 30 min at 8 ºC (Table 10, Figure 10), its resistance to high pressure levels combined with high temperatures was tested, and the results are presented in Figure 11 (see Appendix F for lysozyme activity values). Pressure treatments of 500, 700 and 900 MPa at 40, 60 and 80 ºC for 15 min were performed. Raw milk had a lysozyme activity of 28553 U/mL, which is a little higher than that found for human milk collected from Portuguese women (Table 10). Even though this human milk came from different mothers, the reason for the higher lysozyme concentration may be that this human milk was collected later in lactation, at 6 months.

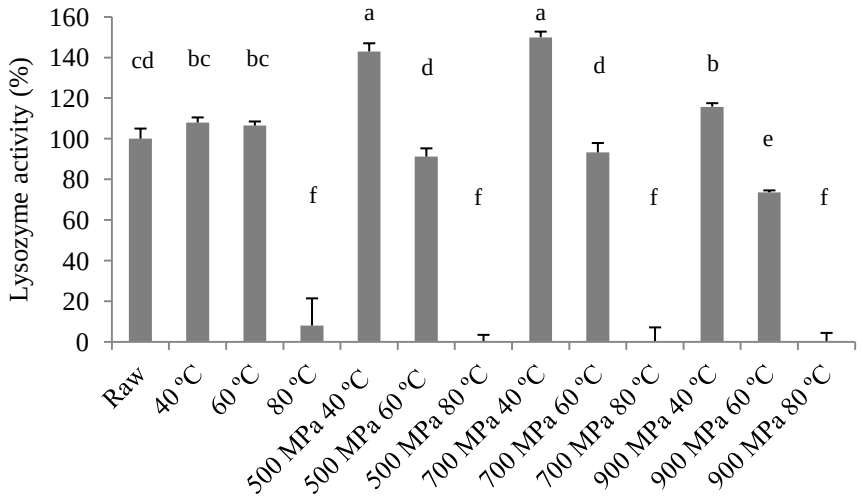

Figure 11. Percentage of lysozyme activity in human milk before and after 15 min exposure to the high temperatures and the HPP treatments performed. Bars with the same letter are not statistically different ($p<0.05$).

The exposure to 40 and 60 ºC did not cause significant differences in lysozyme activity in relation to raw human milk ($p>0.05$, Figure 11). Nevertheless, a 15 min exposure to 80 ºC led to a 92% loss of this enzyme activity (8% retention). This is in agreement with the fact that lysozyme reaches a maximum activity around 50 ºC, and is greatly decreased at 80 ºC (Hayashi *et al.*, 1968). After the pressure treatments at 40 ºC (500, 700 and 900 MPa) lysozyme activity significantly increased to 143, 150 and 116, respectively. This pattern of enzyme activity increase at high temperatures has been reported in literature for other enzymes. For instance, Shook *et al.* (2001) noted that pectinesterase activity in diced tomato was increased after 400 MPa at 45 ºC for 5 min, and Van Den Broeck *et al.* (2000) found that at low pressures and elevated temperatures (100-300 MPa and 60-65 ºC) there was an activation of tomato PME. In this work, after 500 and 700 MPa at 60 ºC lysozyme activity was not significantly inactivated ($p>0.05$). However, when increasing the

pressure level to 900 MPa lysozyme activity fell to 74% (26% loss), which is a significant decrease. HPP at 500, 700 and 900 MPa at 80 ºC reduced lysozyme activity to residual levels (about 0.4%), that is, resulted in almost total inactivation.

In short, pressure treatments at 500, 700 and 900 MPa at 40 ºC increase lysozyme activity in human milk, mostly pressure levels of 500 and 700 MPa. Therefore, it can be asserted that the combined effect of high pressures and moderate temperature effectively increases lysozyme activity. However, the same pressure levels at 60 ºC do not exert that effect anymore. HPP at 900 MPa at 60 ºC even causes a significant reduction in its activity. HPP at 80 ºC completely inactivates lysozyme.

4. Conclusions

In agreement with what we had reported for human colostrum, IgA content in mature human milk is retained after HPP at 200 and 400 MPa for all holding times, and 600 MPa for 2.5 min. These same treatments are also effective in maintaining IgG concentration. On the other hand, HPP treatments at 600 MPa for 15 and 30 min, and Holder pasteurisation resulted in similar significant decreases of these Igs (up to 31% for both). IgM, in its turn, was not significantly decreased after 200 and 400 MPa for 2.5, 15 and 30 min. Nevertheless, a significant 37% decrease after 600 MPa for 2.5 min was observed. Still, this decrease is much lower than that caused by Holder pasteurisation, of 69%. HPP at 600 MPa for 15 and 30 min caused marked decreases, which were not significantly different from those resulting of pasteurisation treatment.

Regarding antimicrobial enzymes, lactoperoxidase activity was not found. Lysozyme activity was significantly decreased to 50% after Holder pasteurisation, but maintained or increased after all of the HPP treatments at 8 ºC. Treatments of 200 MPa for 15 min, 400 MPa for 30 min, and 600 MPa for 15 min led to 111, 118 and 110% retentions, which suggests that some pressure levels and holding times may cause lysozyme activation. When combining HPP with high temperatures, we observed that after 500, 700 and 900 MPa at 40 ºC lysozyme activity was also significantly increased to 143, 150 and 116%, respectively. The increases at 500 and 700 MPa at 40 ºC are the highest found in this work. After 500 and 700 MPa at 60 ºC its activity was not significantly different from that of raw milk, but 900 MPa at 60 ºC already caused a significant 26% inactivation. After HPP at 80 ºC the enzyme was almost completely inactivated, and only presented residual activity.

These findings indicate that not only HPP treatments at 200 and 400 MPa up to 30 min are a better alternative to Holder pasteurisation in terms of Ig and lysozyme retention, that also some of these treatments may induce an increase in the available Ig content and lysozyme activity in human milk. HPP treatments at 500, 700 and 900 MPa at 40 ºC proved to be effective in activating lysozyme, as well. Thus, other studies should be conducted in order to determine HPP effects in other nutritional and non-nutritional human milk components.

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CHAPTER 4

General Conclusions and Future work: A Synopsis of Future Requirements

The immunological and functional benefits that only human milk provides to infants have led to an increased interest in banked donor human milk. Nowadays, there is no doubt left that donor human milk is definitely a better choice than cow's milk-based infant formulas. However, the pasteurisation method that is currently applied to assure the safety of donor milk provided by HMB is far from being ideal. Holder pasteurisation is responsible for considerable decreases in the antimicrobial and functional biomolecules that make human milk the optimum nutritional source for infants. Therefore, to study other pasteurisation technologies that simultaneously minimise microbial contaminants and maintain these factors is critical for the development of safe effective milk banking procedures. HPP is par excellence one of those technologies and, probably, the most promising one.

HPP is, however, in a very premature stage of investigation in what concerns human milk pasteurisation and preservation, and few studies have been conducted so far (Table 7). In the present work a comprehensive review of human milk composition and preservation in HMB, as well as HPP principles, operation and reported effects in human milk was carried out in order to help understand what is already known and the best way to apply this promising pasteurisation technology to such a valuable food. Furthermore, this work evaluated HPP effects on IgA, IgM, and IgG contents, and lysozyme activity in human colostrum and milk, as lactoperoxidase activity was not detected. It was concluded that, when comparing HPP with Holder pasteurisation, HPP at 200 and 400 MPa resulted in much higher retentions of these antimicrobial factors in human colostrum and milk. In almost all of the components there were no significant differences between pressure processed and raw human milk samples (excepting IgM in mature milk after 400 MPa for 15 and 30 min), while Holder pasteurisation always caused significant decreases. These results show the great potential of HPP to be used in HMB.

Notwithstanding, there is still a long path to be taken before HPP can be developed into a successful pasteurisation technology for human milk. Regarding human milk components, HPP effects in the overall nutritional composition have to be assessed. It is essential to study HPP effects in human milk lipids, as well as fatty acid profile; whey proteins and casein; carbohydrates in general (mainly lactose) and oligosaccharides in particular, as they have important bioactive functions. Besides, its effects on vitamins and minerals also have to be determined. HPP will only be a viable alternative if high pressure does not affect these nutritional components. Further work should also focus on investigating HPP effects on other bioactive and immune factors (namely

those decreased by Holder pasteurisation), such as lactoferrin, both lipases (BSSL and LPL), amylase, lymphocytes, cytokines and growth factors.

In what concerns human milk contamination with microorganisms, research is needed to evaluate HPP capacity to effectively inactivate relevant bacterial and viral pathogens. Even though some work has been done for determining HPP effects on some vegetative bacteria with interesting results, more studies are required to establish recommended processing times that result in satisfactory inactivation of all relevant pathogens, and maintain human milk natural composition. For example, studies of HPP inactivation of bacteria in the bacillus species, as *Bacillus cereus*, are fundamental. Studies on viral pathogens are essential as well, and possible viral pathogens to study include HIV, HTLV, polivirus, herpes simplex virus, hepatitis A, and cytomegalovirus. Moreover, antimicrobial compounds present in human milk should be further investigated in combination with HPP to clarify a possible synergy that promotes higher inactivation of pathogens.

APPENDICES

Appendix A. Informed consent given to all of the participant mothers.

CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO PARA

PARTICIPAÇÃO EM PROJETO DE INVESTIGAÇÃO

O meu nome é Maria Sílvia Gomes de Sousa e estou a realizar a minha tese de Mestrado em Biotecnologia, ramo Alimentar, inserida num grupo de investigação do Departamento de Química da Universidade de Aveiro, sendo orientada pelo Prof. Dr. Jorge Manuel Alexandre Saraiva, professor na mesma instituição de ensino. Estamos a desenvolver um projeto intitulado "Efeito da alta pressão em leite e colostro humanos com vista à sua pasteurização atérmica/a frio" que visa o estudo de um novo método de pasteurização do leite materno – a alta pressão – que permite melhor conservar o seu valor nutricional comparativamente àquele usado atualmente em bancos de leite materno – a pasteurização térmica –.

Para desenvolver este método alternativo de pasteurização atérmica por alta pressão, o qual garante a segurança microbiológica do leite materno com maior manutenção das suas propriedades nutricionais, solicitamos a sua participação no estudo através da doação do leite ou colostro que não necessite para a nutrição do seu bebé, agradecendo desde já a colaboração.

Abaixo esclarecem-se algumas questões bastante pertinentes:

Porquê? Os bancos de leite humano são de extrema importância para as mães que, por alguma razão, não podem amamentar ou não possuem leite suficiente para suprir as necessidades do seu bebé, uma vez que este apresenta benefícios imunológicos e nutricionais acrescidos relativamente às fórmulas infantis à base de leite de vaca, diminuindo consideravelmente o risco de ocorrência de infeções e diversas doenças crónicas. O método de pasteurização térmica comummente usado garante a segurança microbiológica do leite, mas produz um leite "cozido" com características nutricionais e funcionais diminuídas em relação ao leite "cru" não pasteurizado, bem como alterações nas propriedades sensoriais. Assim, o leite de mães dadoras pasteurizado não é suficiente para garantir a satisfação das necessidades nutricionais e imunológicas dos bebés, principalmente daqueles nascidos prematuramente, doentes ou com peso muito baixo, os quais apresentam maiores carências nutricionais. É então fulcral a evolução dos estudos de pasteurização atérmica do leite e colostro humanos, alimentos essenciais ao saudável crescimento e desenvolvimento dos bebés.

Procedimentos? Será realizada a recolha de amostras de leite materno de acordo com as seguintes etapas:

1) Pedido de consentimento informado, livre e esclarecido para a participação neste projeto de investigação. É indispensável o consentimento de todas as mães participantes para a recolha do seu leite.

2) Extração do leite materno através do uso de bombas adequadas para esse fim, recolha do mesmo para frascos estéreis de plástico e preenchimento da ficha de registo da(s) data(s) de recolha do leite. Esta etapa do processo será realizada pelas mães preferencialmente e sempre que possível no hospital ou então em casa.

Riscos? Não existem quaisquer riscos ou ameaças à integridade física ou emocional das participantes. A extração do leite é segura, indolor e não se conhecem efeitos secundários.

Benefícios? A oportunidade de colaborar num projeto pioneiro de estudo de uma nova e promissora tecnologia de pasteurização atérmica que pode melhorar consideravelmente a qualidade nutricional e sensorial do leite de mães dadoras disponível nos bancos de leite humano, ajudando muitos bebés. Todas as mães participantes terão acesso aos resultados e conclusões do projeto, caso assim o desejem.

Alternativas? A participação no projeto através da doação de leite materno é voluntária, podendo solicitar todos os esclarecimentos que considere necessários. Para participar apenas tem de efectuar os procedimentos descritos acima. Tem igualmente a opção de não participar ou interromper a participação em qualquer altura, não sendo adotados quaisquer procedimentos discriminatórios na sua assistência nesta Unidade de Saúde.

Dúvidas? Qualquer dúvida que surja pode e deve ser colocada em qualquer altura através do meu contacto telefónico ou e-mail, 917605872 e silviasousa@ua.pt, respetivamente. Abaixo esclarecem-se algumas dúvidas frequentes:

- A sua participação não lhe trará nenhum tipo de custos;

- O leite doado será usado exclusivamente no âmbito deste projeto de investigação;

- É garantida confidencialidade, sendo que todos os dados das mães participantes serão apenas conhecidos pela equipa médica e de investigação;

- Os resultados do estudo serão publicados em revista científica e na minha tese de Mestrado.

[ver continuação na página seguinte deste documento]

Confirmo que expliquei à pessoa abaixo indicada, de forma adequada e inteligível, os procedimentos necessários ao ato referido nas páginas 1 e 2 deste documento. Respondi a todas as questões que me foram colocadas e assegurei-me de que houve um período de reflexão suficiente para a tomada da decisão. Também garanti que, em caso de recusa, não serão adotados quaisquer procedimentos discriminatórios no contexto da sua assistência nesta Unidade de Saúde.

O pedido de assinatura deste documento resulta do disposto na Circular Normativa nº 2 /DSMIA de 15/01/2007, da Direção-Geral da Saúde.

Nome legível do/a médico/a ou investigador/a: |____________________________________|

Data … …/… …/… … … Assinatura ….

-+-

Por favor, leia com atenção todo o conteúdo deste documento. Não hesite em solicitar mais informações se não estiver completamente esclarecida. Verifique se todas as informações estão corretas. Se tudo estiver conforme, então assine este documento.

"Declaro que concordo com a doação do meu leite materno conforme me foi proposto e explicado pela pessoa que assina este documento (para além de mim), conhecendo o seu fim, procedimentos a seguir, riscos, benefícios e alternativas e tendo podido fazer todas as perguntas sobre o assunto. Assim, autorizo a realização do ato indicado nas condições em que me foram explicadas e constam deste documento."

… … … … … … … … … … (local), … …/… …/… … … (data)

Nome: |____|

Assinatura ✗ …

Se não for a própria a assinar por incapacidade comprovada ou idade abaixo de 16 anos:
Nome: ....
B.I./C.C. Nº: .... datado de ...../...../......, validade …../…../…...
Grau de parentesco ou tipo de representação: ....
Assinatura ✗ …

Referências:
Documento-Guia sobre Consentimento Informado
http://portal.arsnorte.min-saude.pt/portal/page/portal/ARSNorte/Comiss%C3%A3o%20de%20%C3%89tica/Ficheiros/Consentimento_Informado_Doc_Guia.pdf
Modelos de Consentimento Informado
http://portal.arsnorte.min-saude.pt/portal/page/portal/ARSNorte/Comiss%C3%A3o%20de%20%C3%89tica/Ficheiros/Consentimento%20Informado/7A4D4ADDB2579BA4E040140A110272B3

Muito obrigada pela participação!

Appendix B. Standard curves constructed for the determination of immunoglobulins in human colostrum and mature milk.

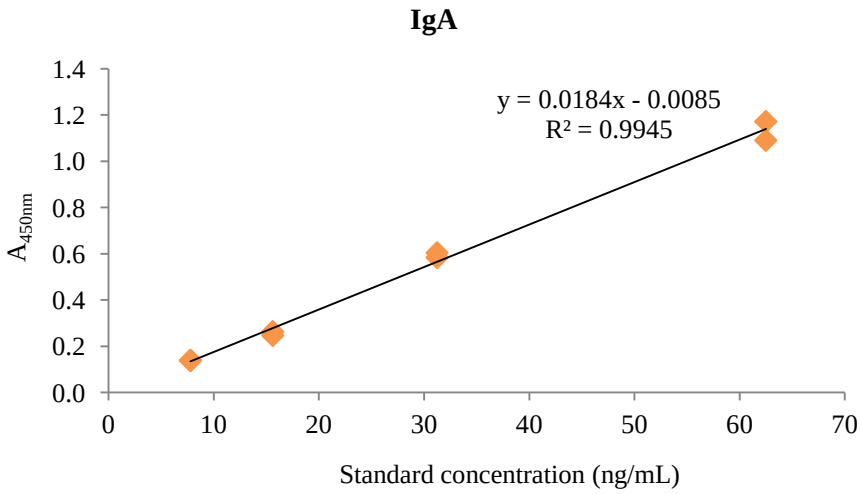

Figure B1. Standard curve used for determining IgA concentration in human colostrum and mature milk.

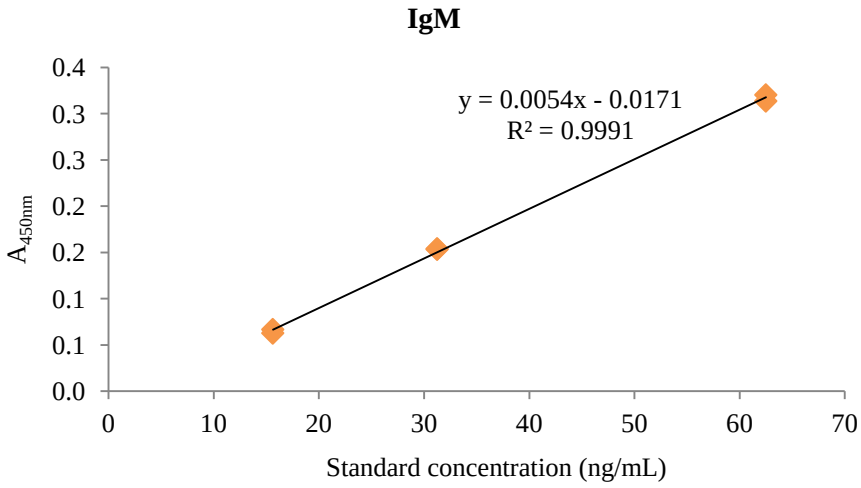

Figure B2. Standard curve used for determining IgM concentration in human colostrum and mature milk.

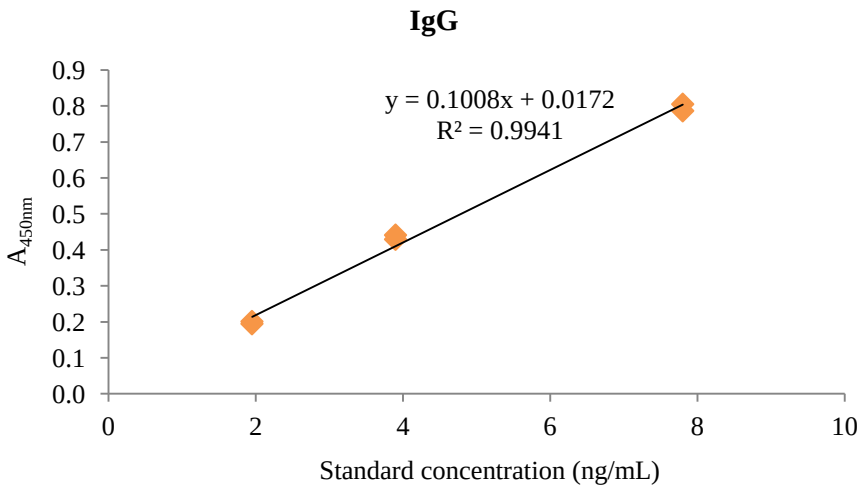

Figure B3. Standard curve used for determining IgG concentration in human colostrum and mature milk.

Appendix C. Immunoglobulin concentration and lysozyme activity in raw and treated colostrum samples.

Table C1. Colostral IgA, IgM and IgG concentrations and lysozyme activity before and after Holder pasteurisation and the various HPP treatments.

Treatment	IgA concentration (mg/mL)	IgM concentration (mg/mL)	IgG concentration (mg/mL)	Lysozyme activity (U/mL)
Raw	1.728 ± 0.034	0.200 ± 0.011	0.199 ± 0.010	18033 ± 570
Holder	1.383 ± 0.026	0.098 ± 0.002	0.152 ± 0.001	10127 ± 286
200 MPa 2.5’	1.701 ± 0.063	0.201 ± 0.006	0.193 ± 0.003	18040 ± 0
200 MPa 15’	1.740 ± 0.082	0.196 ± 0.017	0.207 ± 0.001	16667 ± 726
200 MPa 30’	1.733 ± 0.015	0.219 ± 0.017	0.180 ± 0.004	17020 ± 632
400 MPa 2.5’	1.782 ± 0.006	0.215 ± 0.007	0.184 ± 0.001	18687 ± 1588
400 MPa 15’	1.739 ± 0.007	0.201 ± 0.008	0.182 ± 0.006	16720 ± 635
400 MPa 30’	1.722 ±0.053	0.187 ± 0.013	0.182 ± 0.004	18900 ± 311
600 MPa 2.5’	1.669 ± 0.064	0.157 ± 0.003	0.157 ± 0.008	18460 ± 0.728
600 MPa 15’	1.388 ± 0.022	0.081 ± 0.001	0.129 ± 0.004	17580 ± 347
600 MPa 30’	1.284 ± 0.000	0.080 ± 0.004	0.120 ± 0.010	18080 ± 673

Appendix D. First order kinetic denaturation of human colostrum immunoglobulins.

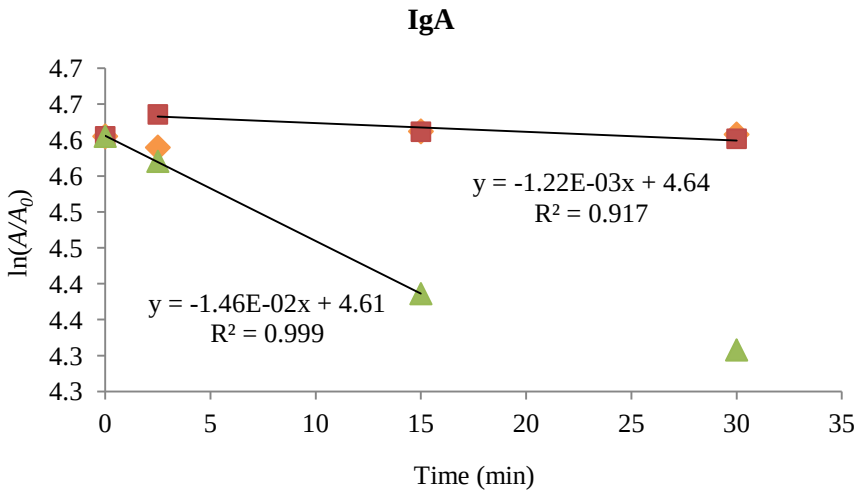

Figure D1. Effect of pressure treatment on human colostrum IgA concentration (A/A_0) as a function of treatment time at different pressures: 200 MPa (◆), 400 MPa (■), 600 MPa (▲).

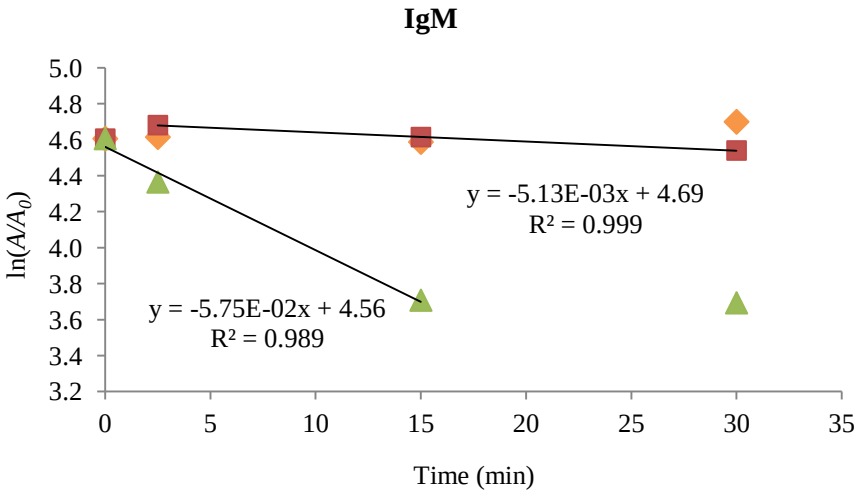

Figure D2. Effect of pressure treatment on human colostrum IgM concentration (A/A_0) as a function of treatment time at different pressures: 200 MPa (◆), 400 MPa (■), 600 MPa (▲).

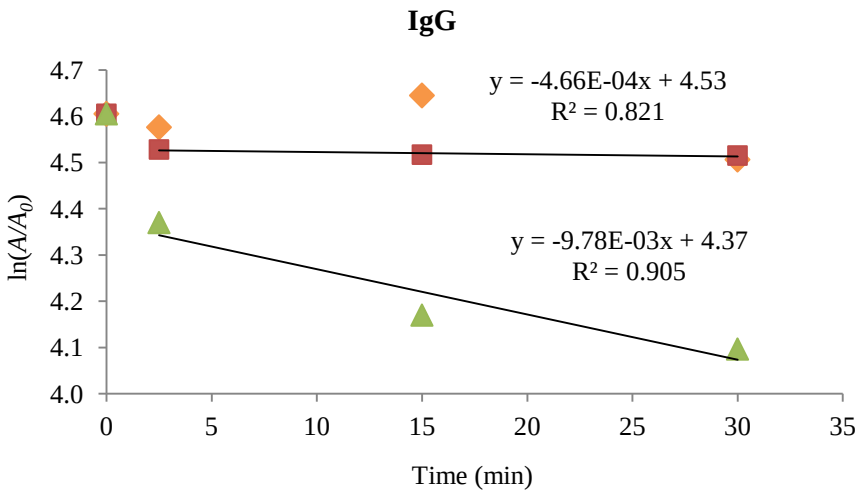

Figure D3. Effect of pressure treatment on human colostrum IgG concentration (A/A_0) as a function of treatment time at different pressures: 200 MPa (◆), 400 MPa (■), 600 MPa (▲).

Appendix E. First order kinetic denaturation of human milk immunoglobulins.

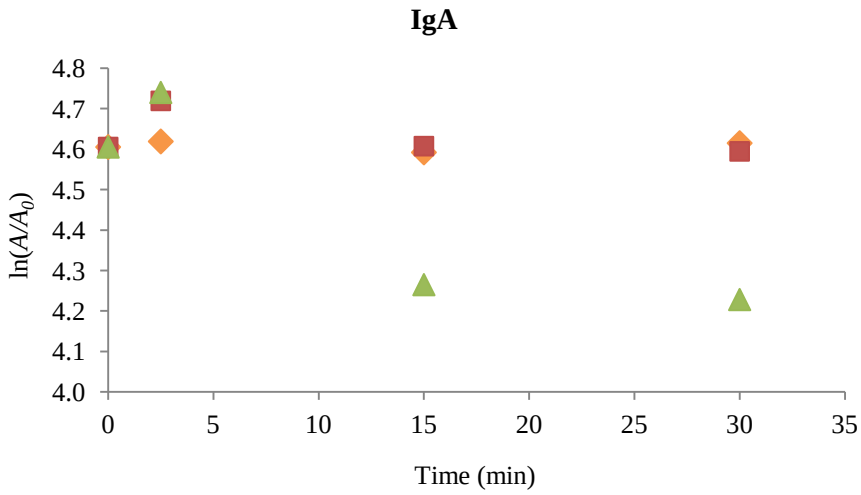

Figure E1. Effect of pressure treatment on human milk IgA concentration (A/A_0) as a function of treatment time at different pressures: 200 MPa (◆), 400 MPa (■), 600 MPa (▲).

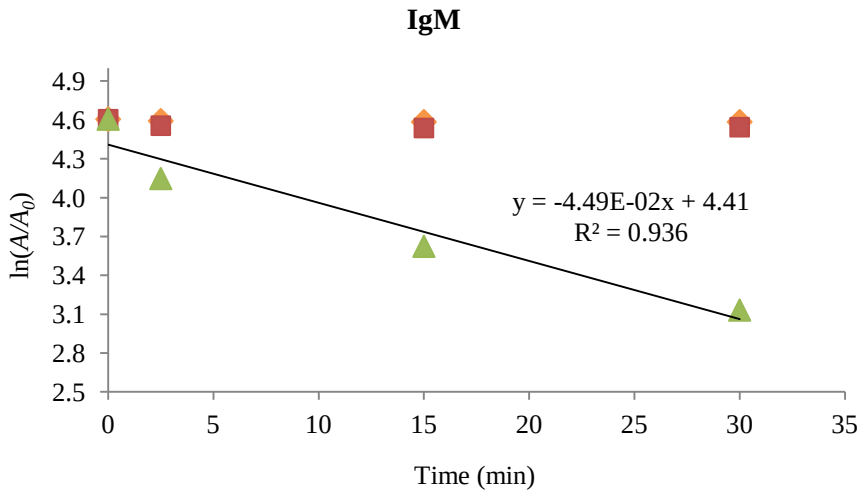

Figure E2. Effect of pressure treatment on human milk IgM concentration (A/A_0) as a function of treatment time at different pressures: 200 MPa (◆), 400 MPa (■), 600 MPa (▲).

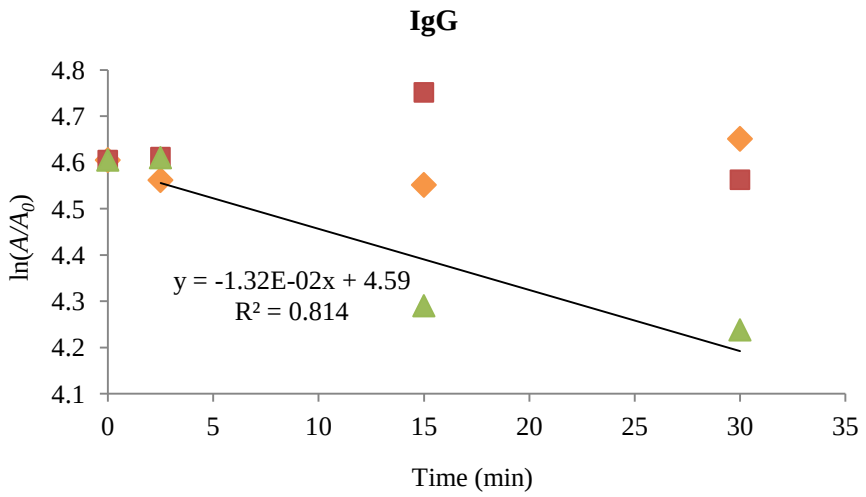

Figure E3. Effect of pressure treatment on human milk IgG concentration (A/A_0) as a function of treatment time at different pressures: 200 MPa (◆), 400 MPa (■), 600 MPa (▲).

Appendix F. Lysozyme activity in raw and processed human milk samples (HPP at high temperatures).

Table F 1. Lysozyme activity in human milk before and after 15 min exposure to the high temperatures and HPP treatments performed.

Treatment	Lysozyme activity (U/mL)
Raw	28553 ± 1428
40 ºC	30820 ± 780
60 ºC	30397 ± 613
80 ºC	2290 ± 306
500 MPa 40 ºC	40813 ± 1641
500 MPa 60 ºC	26040 ± 1054
500 MPa 80 ºC	118 ± 4
700 MPa 40 ºC	42807 ± 1192
700 MPa 60 ºC	26627 ± 1227
700 MPa 80 ºC	105 ± 7
900 MPa 40 ºC	33040 ± 584
900 MPa 60 ºC	21000 ± 209
900 MPa 80 ºC	77 ± 3