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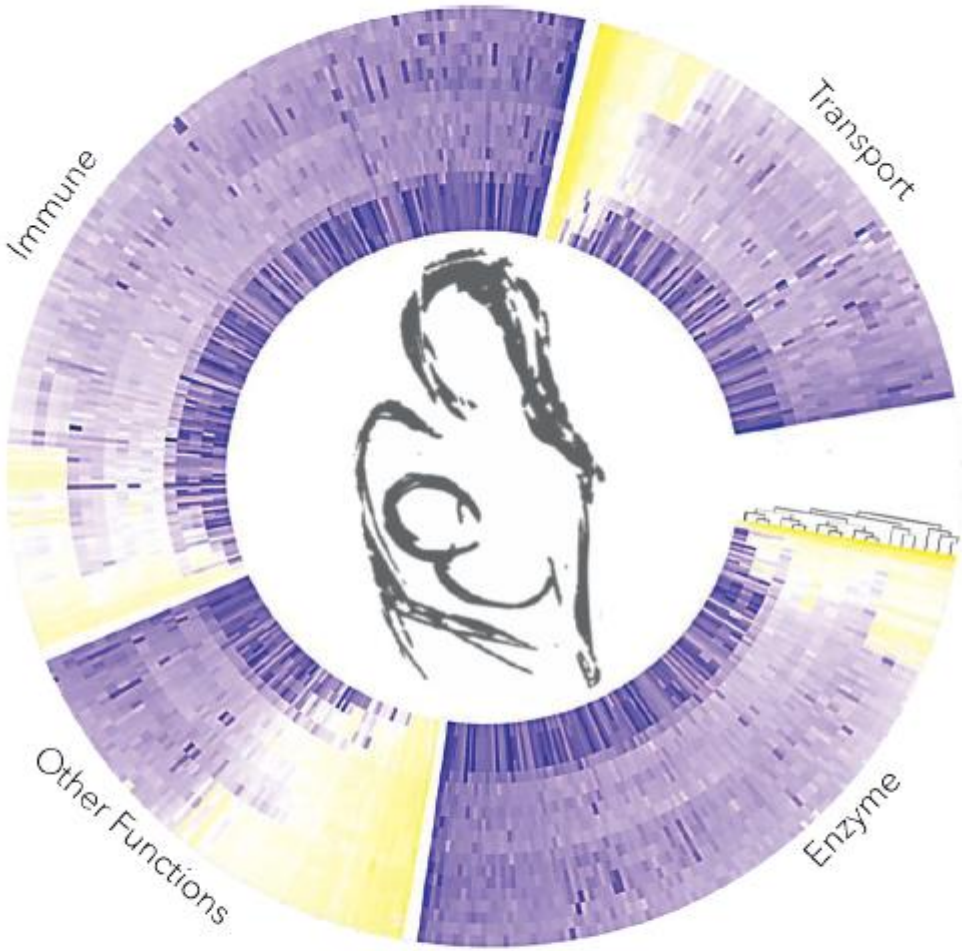

IMPROVING DONOR HUMAN MILK BY NOVEL PROCESSING TECHNOLOGIES

EVA KONTOPODI

Improving donor human milk by novel processing technologies

Eva Kontopodi

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Improving donor human milk by novel processing technologies

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TABLE OF CONTENTS

Chapter 1	General introduction	3
Chapter 2	Testing the Effects of Processing on Donor Human Milk: Analytical Methods *(Published in Food Chemistry)*	19
Chapter 3	"Donor milk banking: Improving the future". A survey on the operation of the European donor human milk banks *(Published in PlosONE)*	43
Chapter 4	High–Temperature Short–Time preserves human milk's bioactive proteins and their function better than pasteurization techniques with long processing times *(Published in Frontiers in Pediatrics)*	63
Chapter 5	Effects of High–Pressure Processing, UV–C irradiation and thermoultrasonication on donor human milk safety and quality *(Accepted in Frontiers in Pediatrics)*	85
Chapter 6	Thermoultrasonication, ultraviolet–C irradiation, and high–pressure processing: Novel techniques to preserve insulin in donor human milk *(Published in Clinical Nutrition)*	107
Chapter 7	Human Milk from Previously COVID–19–Infected Mothers: The Effect of Pasteurization on Specific Antibodies and Neutralization Capacity *(Published in Nutrients)*	115
Chapter 8	Processing methods of donor human milk evaluated by measuring milk procoagulant activity *(Published in Innovative Food Science & Emerging Technologies)*	133
Chapter 9	General discussion	145
Appendices	English summary	166
	Nederlandse samenvatting	167
	PhD portfolio	168
	Contributing authors	171
	Acknowledgments	173

Chapter 1

General Introduction

1.1. Vulnerable infants and human milk

According to WHO, around 15 million babies are born preterm, which is regarded as the leading death cause among newborns [1]. Preterm infants are infants born before 37 weeks of gestation, while infants born between 28 to 32 weeks of gestation are classified as very preterm [1, 2]. Low birth weight is defined as a weight less than 2500 g, while infants with a birth weight less than 1500 gr are considered of very low birth weight [2]. Infants that fall into the aforementioned categories are at increased risk of early growth retardation, developmental delay, long–term ill–health, and even death [2, 3]. For these vulnerable newborns, human milk (HM) is of crucial importance, as it leads to lower mortality rates and significantly reduces the risk of necrotizing enterocolitis (NEC) [4, 5]. In addition, HM offers enormous benefits to all infants, as it supports gut maturation, host defence, cognitive development, metabolic and cardiovascular health, and is generally associated with better health during adulthood [3–5]. HM is generally regarded as a dynamic biological system, tailored to every infant's needs, nutritionally, immunologically and developmentally [6–9]. For that reason, exclusive HM feeding for at least the first six months of life is considered as the standard for infant nutrition [9, 10].

Mother's own milk should always be the first choice for infant feeding [2, 6, 11]. When mother's own milk is unavailable, official policy bodies such as the World Health Organization (WHO), the American Academy of Pediatrics (AAP), and the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) recommend donor HM (DHM) as the best alternative for infant feeding [6, 12]. In cases where neither mother's own milk nor DHM are available, preterm formula should be used [12]. The major advantage of DHM over preterm formula is the decreased risk of NEC [12, 13].

1.2. Human milk banking

DHM should be dispensed only through established human milk banks (HMBs) that can ensure its safety and optimize its composition by implementing all necessary measures and control systems [3, 6]. The role of HMBs is to collect DHM from donors that are first selected via a thorough screening process and then screen, store, process and distribute this milk to vulnerable infants [6, 14]. The first HMB opened in Vienna in 1909 and the second one opened in Boston a year later [14]. Currently, there are more than 750 active HMBs worldwide and 280 of those HMBs are located in Europe [15]. All practices carried out by these HMBs are regulated by specific guidelines or locally implemented standards [13, 16]. **Figure 1** offers an overview of the practices followed.

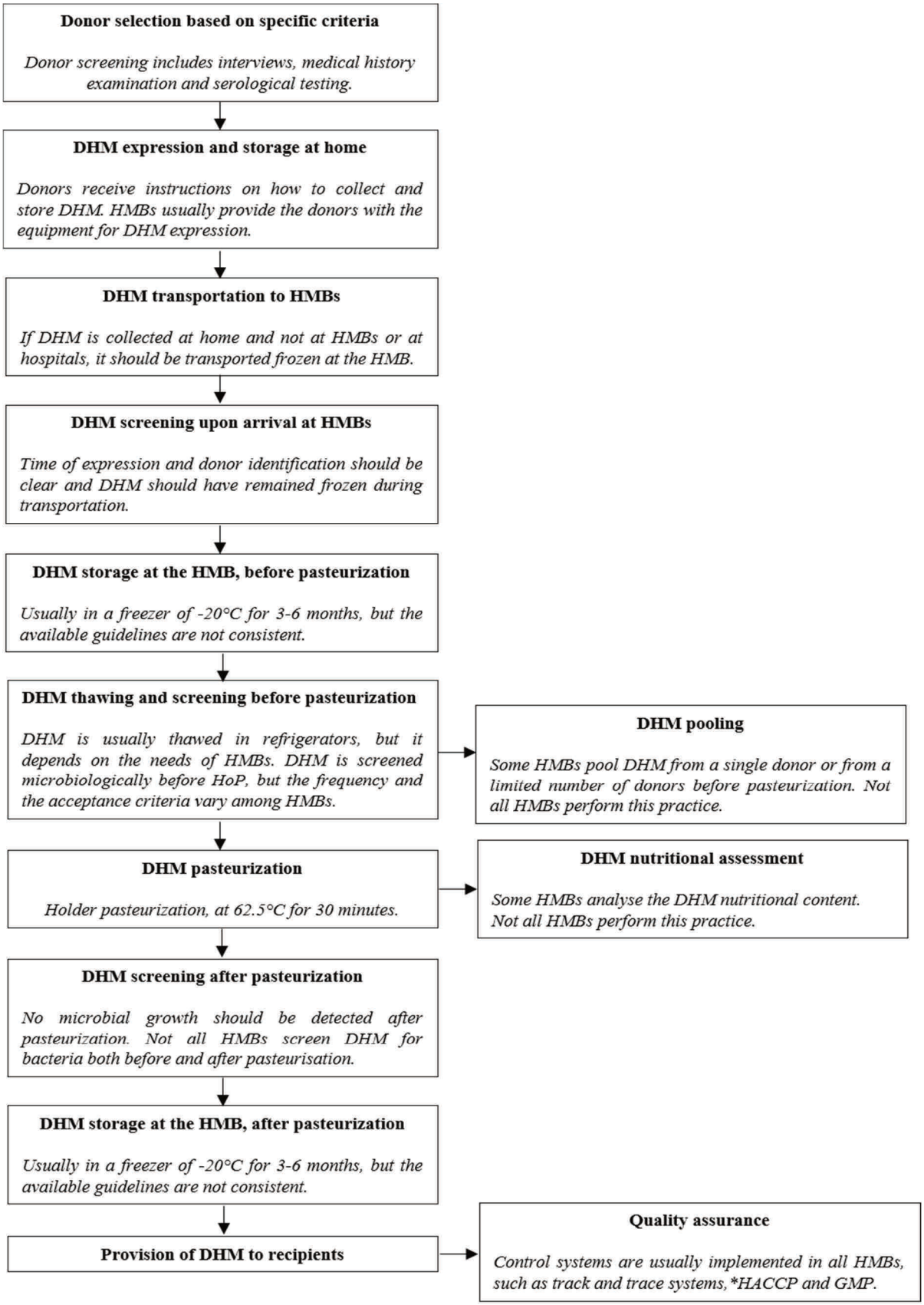

Figure 1. Practices followed in HMBs. * Hazard Analysis and Critical Control Points (HACCP) and Good Manufacturing Process (GMP).

1.2.1. DHM contamination

Whether it's mother's own milk or DHM, contamination with pathogenic or opportunistic pathogenic bacteria is a common concern [17]. The most frequently isolated pathogenic species belong to *Staphylococcus* and *Enterobacteriaceae* species [18, 19]. Other pathogenic species of concern include group B *Streptococcus* and *Bacillus* species that can lead to neonatal sepsis [20, 21]. These contaminants may be introduced in HM through certain practices during HM handling both by donors and by HMBs, such as collection, storage, transportation, thawing, pooling and processing, but they can also be transmitted to HM from the mother's skin or from the infant's oral cavity [21, 22]. As certain bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus cereus* can produce enterotoxins, thermostable enzymes or spores, DHM handling and processing in HMBs should always be well–monitored and quality assurance systems should be implemented [23]. Viral agents may also be found in HM, which are mostly secreted via the mammary tissue, but could also be the result of contamination through skin or from respiratory droplets during milk expression [24, 25]. Cytomegalovirus represents the most frequently detectable virus in HM [26]. To eliminate such microbial contaminants in DHM and to prevent disease transmission from a donor mother to a vulnerable infant, HMBs pasteurize DHM [23]. In addition, HMBs screen DHM for pathogens either post–pasteurization or both pre– and post–pasteurization, but the frequency of testing and the acceptance criteria vary among HMBs [13]. Bacterial and viral inactivation in a HMB setting is further discussed in chapter 2.

1.1.DHM pasteurization

All international milk banking guidelines currently recommend pasteurizing DHM at 62.5°C for 30 minutes, which is the method commonly known as holder pasteurization (HoP) [27]. According to the guidelines, HoP offers a compromise between the microbiological safety and the quality of DHM; most pathogens and a number of viruses (e.g. Human immunodeficiency virus, human T–lymphotropic virus, cytomegalovirus, herpes and rubella) are inactivated, several nutritional factors (e.g. lactose, human milk oligosaccharides (HMOs), fatty acids and fat soluble vitamins) are preserved, but key bioactive components such as immunoglobulins, a number of enzymes, hormones, growth factors, cytokines, cellular components and heat labile vitamins are greatly reduced [8, 23, 27, 28]. In practice, this degradation may lead to a loss of the milk's health benefits for the neonate. For example, the complete elimination of bile salt–stimulated lipase (BSSL) after HoP, which is an enzyme essential for fat digestion and absorption, could be the primary cause of the lower growth rates of preterm infants fed pasteurized DHM when compared to raw DHM [29]. For such reasons, there's a great interest in optimizing DHM processing [8, 12, 27].

According to milk banking guidelines, the ideal pasteurization process includes a phase where DHM is heated up rapidly, then a phase where the temperature remains constant, and finally, a phase where DHM is rapidly cooled down [27]. Many parameters can affect these phases, such as the equipment used, the heating medium, the volume of milk included in a pasteurization cycle, the heat exchange surface to milk volume ratio and the heat transfer rate of DHM or of the bottle used [30]. However, guidelines on the full temperature profile that would include heating up and cooling down durations are currently not available. Nevertheless,

ensuring the optimal pasteurization performance is of crucial importance, since the duration of the thermal treatment and the temperature that DHM is being exposed to, can have a direct effect on the preservation of its bioactive components. In fact, key immunological components in DHM such as secretory immunoglobulin A (sIgA), lactoferrin and lysozyme were shown to be significantly reduced already at 58°C, while BSSL inactivation has been reported to start at around 45°C [27, 31, 32]. Therefore, the time during which DHM is exposed to high temperatures should be minimized to reduce the damage of such components.

1.3. DHM functionality

Most nutrients are not negatively affected by HoP, but significant losses of a large number of DHM functional components have been reported [8, 28, 33]. A high retention of the functional DHM components after processing is desirable, in order to support the infant's physiological development and to enhance its immature host defence system. The provision of maternal milk antibodies to the neonate is such an example, which compensates for the insufficient amounts of antibodies produced by the immature immune system of preterm infants, thus protecting them from infections [34]. In general, various HM components are shown to be bioactive; HMOs, long–chain polyunsaturated fatty acids (PUFA), antioxidants, cellular components and a great number of proteins are included among these bioactive components [35]. For instance, HMOs are known to act as prebiotics, antimicrobials, immunomodulators and also to support brain development, and since specific HMOs functions have been associated with specific HMOs structures, it's important to determine their structure and the concentration of those structures [36–38]. For these components to exert their bioactivities in an infant's gastrointestinal tract, they need to be resistant or relatively resistant against proteolytic degradation and retain an intact or partially intact structure, for at least some time during the digestive process. Practically, they need to survive through the stomach's low pH and pepsin activity as well as the activities of pancreatic enzymes, at least at a certain degree [35, 39]. Of the aforementioned bioactive components, the ones most significantly affected by HoP are the HM proteins [28]. For that reason, the focus of the current thesis is set on the most affected bioactive proteins.

1.3.1. DHM functional proteins

Proteins exert the largest variety of bioactivities in HM [35]. Mature HM contains approximately 8–11 g/L of total protein, which decreases as lactation progresses. The proteins in HM are divided into caseins, serum and milk fat globule membrane proteins. Colostrum, which is the milk produced up to five days after delivery, has a 90:10 serum–to–casein ratio, while this ratio is 60:40 in mature HM [7, 40]. Serum proteins are soluble, whereas caseins are suspended in solution, in the form of casein micelles [7]. Approximately 13% of the total HM protein content is casein, with β–casein being the most predominant [7]. In the serum fraction, α–lactalbumin is the most abundant protein, followed by lactoferrin, sIgA, lysozyme, and serum albumin [9, 40].

The resistance towards complete proteolysis was first shown for sIgA, which was found in intact form in the stool of infants fed HM [39]. The increased resistance of sIgA against trypsin and pepsin digestion, is mainly attributed to the attachment of secretory components to

the dimeric backbone of sIgA [41]. SIgA binds to and neutralizes invasive pathogens at the mucosal surface, in order to prevent their adherence to epithelial cells and protect the infant from many different enteric and respiratory infections [7, 34]. SIgA concentrations are the highest in colostrum (about 12 mg/ml), while in mature HM only amounts of approximately 1 mg/ml can be found [7]. The decrease in its levels over the course of lactation reflects the infants' needs, as their immune system matures [7, 9]. HM IgM and IgG may also play a role in protecting the infant against infections at the mucosal surface, but their concentrations are much lower [34, 42].

Significant quantities of lactoferrin, which is another major protein in HM, have been also found intact in the stool of infants, especially during early infancy [43]. Colostrum contains very high quantities of lactoferrin (about 10 mg/ml), which is an indicator of the important activities of this protein in the neonatal period [43]. Lactoferrin's bacteriostatic activity against *Escherichia coli* was one of the first bioactive functions attributed to a HM protein [35]. Lactoferrin is mainly present in an unsaturated form, with one single polypeptide chain comprised by two homologous domains, each able to bind to one ferric and one carbonate ion [44, 45]. Due to its high iron binding affinity, lactoferrin withholds iron from iron–dependent pathogens such as *E. coli,* thus reducing their growth [45]. Through this mechanism, this protein can inhibit a variety of both gram negative and positive bacteria [45]. Lactoferrin may also damage the outer bacterial membrane directly, which leads to DNA, RNA and protein synthesis inhibition [40, 45]. Apart of having antibacterial properties, this glycoprotein has many other functions, such as the stimulation of cell differentiation, the iron absorption regulation, as well as anti–inflammatory and immunomodulating activities [43].

Lysozyme is another highly abundant protein in HM that has been found in the stools of infants fed with HM, which suggests resistance against proteolysis [43]. Secreted by polymorphonuclear leukocytes, lysozyme is capable of disrupting the outer cell membrane of gram–positive bacteria, by catalyzing the hydrolysis of the β–1,4 bonds between N–acetylmuramic acid and N–acetylglucosamine residues [40, 46]. A synergistic effect of lactoferrin and lysozyme against gram–negative bacteria *in vitro* has also been reported, with lactoferrin binding to the lipopolysaccharide in the outer bacterial membrane and creating holes through which lysozyme can access the inner proteoglycan matrix and degrade it [35, 46].

HM enzymes, such as BSSL, are essential for digestion in infants, due to their immature digestive systems that are not capable of producing digestive enzymes in adequate amounts [47]. Apart from facilitating lipid digestion and protection of the immature infant intestinal epithelium, BSSL may also exert antimicrobial activities [9]. The antimicrobial mechanisms are mostly attributed to the O–glycosylated repeated motif located at the C–terminal tail of BSSL, which has been shown to bind to dendritic cells [48]. This way, BSSL protects the $CD4^+$ T cells from HIV transinfection, while it also contributes in the elimination of the Norwalk virus [9, 48].

HM additionally contains a number of hormones and growth factors with known bioactive functions, such as gut maturation, regulation of immune responses, immune cell migration and other anti–inflammatory effects [9, 49]. Moreover, HM metabolic hormones such as adiponectin, insulin, leptin and ghrelin are connected with reduced metabolic disease risks, metabolic regulation and inflammation suppression [9]. Insulin in particular is well–connected to intestinal maturation, infant developmental patterns and fat accumulation, while it may

additionally reduce feeding intolerance [50]. The levels of this hormone decrease gradually over the first month of lactation, while it was found to be influenced by maternal insulin sensitivity and BMI [51].

1.3.2. Effects of heat treatment on the DHM functional proteins

Most serum proteins are globular proteins with a complex tertiary structure with numerous disulfide bonds, while caseins lack a well-defined secondary and tertiary structure [52]. Upon heating, the globular serum proteins unfold, a process that includes the breakdown of intramolecular hydrogen bonds. Moving from the native to the denaturated state is usually reversible process, but aggregation is irreversible [53]. These reactions have a direct effect on the protein's biological functions, as these functions are directly correlated with the protein's native three–dimensional structure [54].

HM serum proteins are regarded as fairly heat sensitive, which means that heat exposure may adversely affect their functional properties. Immunoglobulins are such proteins, with some antigen binding sites being more heat labile than other sites [54]. Heat–induced denaturation of HM immunoglobulins have been documented both after HoP and after high–temperature short–time pasteurization (HTST) [55]. In general, HoP has been shown to cause a reduction as high as 88% of sIgA, lactoferrin and lysozyme, although with significant variation among the published literature [31, 56]. This variation can be further seen in **Table 1**, which provides a summary of the available data on the effect of HoP on functional components. Of the different antibody classes found in HM, sIgA seems to be more stable than IgG and IgM during HoP. Lactoferrin's thermal stability is well–correlated with its iron saturation due to the formation of a more rigid tertiary structure, but HoP significantly reduces its functionality [57–59]. The studies available with regards to the effect of HoP on lysozyme show contradictory results (**Table 1**). This variation may be explained by the use of different assays, such as ELISA, RIA and *Micrococcus lysodeikticus*–based turbidimetric assays [28]. Apart from the differences in the analytical methods used, the way HoP is performed among the various studies may additionally contribute to the observed variability [60].

1.4. Alternative methods to HoP for DHM processing

Due to the detrimental effects of HoP on important functional DHM components, the need to improve DHM processing is a critical issue. For that reason, both thermal and non–thermal alternative methods are currently being investigated [8]. The main processing methods taken into consideration include HTST, high–pressure processing (HPP), ultraviolet (UV)–C irradiation and thermo–ultrasonic processing (TUS) [8, 27].

HTST is a well–established method in the dairy industry. During HTST, a thin layer of milk is exposed to 72°C for 15 sec in a continuous flow system [8]. This method is usually preferred in the dairy industry, due to lower energy consumption and faster treatment times [27]. Although HTST systems tailored for use in HMB are not commercially available yet, small–scale HTST pasteurizers have recently been developed and validated for HM processing [27, 61]. HTST was proven to be effective in eliminating bacteria and certain viruses in DHM, such as HIV, CMV, hepatitis B and C [62, 63]. When compared to HoP, HTST showed better results with regards to the antioxidant capacity, lactoferrin, some vitamins (B and C) and

cytokines, but the data on the immunoglobulin, lysozyme and BSSL retention are divergent [8]. Various reasons may contribute to these differences, including the different HTST parameters applied and different technical aspects of the HTST devices used [8, 60].

Based on HTST, flash–heating (FH) was developed, for use in low–income countries [64, 65]. FH is an inexpensive and relatively simple method, usually performed by placing a glass container with DHM in an aluminium pan with water, which is then placed on a hot plate. When the water temperature reaches 100°C, DHM is removed from the pan and is immediately cooled down [64].

HPP is a mild processing method commonly applied in the food industry to provide microbiologically safe and high–quality products [58]. This method includes exposing a packaged sample to high hydrostatic pressure (100–1000 MPa) for a number of minutes [66]. The pressure is evenly distributed throughout the packaged sample, which ensures uniform treatment [67]. Studies that investigated the suitability of this method for DHM processing suggested that HPP may be effective against a number of microbial contaminants, while retaining the DHM bioactive components better than HoP [33]. Nevertheless, differences in HPP sensitivity among bacterial species are reported, while intense pressure conditions may cause similar detrimental effects on the functional DHM components as HoP [8, 27]. With pressures ≤ 400 MPa at room temperature or below, immunoglobulins, lysozyme, some cytokines and vitamins seem to be better retained [8, 33]. However, the wide range of different pressure, time and temperature combinations that have been tested for DHM, make the direct comparison of the available studies difficult [8]. In addition, the scaling down of the HPP equipment for potential use in HMBs, together with the investment and operating costs, remain important obstacles for the utilization of this method [27, 68].

UV–C (200–280 nm), especially between wavelengths of 250 and 270 nm, is highly germicidal against a number of microorganisms [69]. The UV–C–induced microbial inactivation is linked to the photochemical changes that occur within the cell membrane's proteins and nucleic acids due to the absorption of the UV light [70]. This method has been shown to retain the bacteriostatic properties of DHM and to cause no significant losses in BSSL, sIgA, lactoferrin and lysozyme contents, while eliminating a number of vegetative bacteria [30, 69]. Nonetheless, only limited data are currently available to allow for conclusions regarding the suitability of this method for DHM processing [8]. The main issue of this method is that due to high absorption coefficient of HM, the penetration depth of UV–C is limited [69]. To increase UV–C penetration, experimental set–ups that ensure vortical flow have been used with promising results [30, 69, 71]. However, suitable equipment that would enable the application of this method in a HMB context is still lacking.

Ultrasonication (20–100 kHz) is another method used for food preservation in the food industry, which is capable of inactivating a number of bacterial species through the induction of inertial cavitation and sonochemical changes [72]. The microbial inactivation that can be achieved with this method is further enhanced when combined with mild heating (thermoultrasonication, TUS) [73]. However, data regarding the safety of this method and its effect on the DHM bioactive compounds are currently lacking, while the practicality of applying this method in a HMB setting remains an important concern as well.

Table 1. Effects of HoP on HM functional components and the analytical methods used for their determination.

Functional components	Not affected or increased	Decreased by
IgA	**No loss**, electroimmunoassay [74]	**17%**, Photometric quantification [60] **20%**, RIA, chimiluminescence immunoassay, ELISA [59, 63, 75, 76] **26%**, ELISA [77] **27%**, ELISA [78] **28%**, ELISA [31, 55] **30%**, ELISA [79, 80] **35–62%**, RIA [81] **40%**, ELISA [82] **46%**, ELISA [83] **49%**, ELISA [84] **50–55%**, multiplex immunoassay system [85] **51%**, ELISA [30] **54%**, ELISA [61] **50–60%**, multiplex immunoassay system [86] **57%**, ELISA [56]
IgG		**23%**, ELISA [75, 82] **30–40%**, multiplex immunoassay system [86] **30%**, ELISA [76] **34%**, electroimmunoassay [74] **49%**, ELISA [87] **60%**, ELISA [80] **72–79**%, RIA [81]
IgM		**34–37%**, multiplex immunoassay system [85] **50%**, ELISA [82] **51%**, ELISA [75] **80–90%**, multiplex immunoassay system [86] **80%**, ELISA [76] **100% (complete loss)**, RIA [81]
Lactoferrin		**48%**, HPLC [88] **57%**, electroimmunoassay [74] **60%**, ELISA [87] **62%**, RIA [59] **66%**, ELISA [77] **78%**, ELISA [31] **80%**, ELISA [60] **81%**, ELISA [30] **84%**, ELISA [83] **88%**, ELISA [78]
Unsaturated iron–binding capacity		**67–74%**, titration against a ferric nitrilotriacetate solution [59]

Table 1 (*Continued*)

Lysozyme	**No loss**, *M. lysodeikticus* assay [59, 88] **25%**, *M. lysodeikticus* assay [56]	**17%**, ELISA [77] **21%,** *M. lysodeikticus* assay [84] **24%**, electroimmunoassay [74] **26%**, ELISA [83] **35%**, chemiluminescence immunoassay, ELISA [60, 63] **44%**, *M. lysodeikticus* assay [75] **48%**, *M. lysodeikticus* assay[61] **59%**, EIA [30] **61%**, ELISA [31] **65–85%**, lysoplate with *M. lysodeikticus* cells [81]
BSSL		**100% (complete loss)**, chemiluminescence immunoassay, photometric quantification, p–nitrophenol assay [60, 61, 63, 88–91]
Insulin		**13%**, magnetic bead immunoassay [92] **32%**, ELISA [87] **46%**, electrochemiluminescence immunoassay [93]

1.5. Aim and outline of the thesis

DHM represents the best alternative if mother's own milk is unavailable. Most HMBs process DHM with HoP, which ensures microbiological safety but causes a significant decrease in many of its functional components. Since these components are essential for infant health and development, optimization of the DHM biological quality remains a priority [27]. On the basis of these findings, the aim of the current thesis was to evaluate whether alternative methods to HoP are capable of preserving the bioactive DHM components better than HoP, while assuring microbiological safety.

Chapter 2 provides and overview of the analytical techniques available for the evaluation of such novel methods, along with their principles, benefits and drawbacks. An efficient workflow for the analysis of differentially processed DHM is also proposed. **Chapter 3** offers a detailed overview of the actual milk banking practices across Europe, with emphasis on the practices that may have an impact on DHM safety and quality. Thermal processing is further evaluated in **chapter 4**, where the effects of various processing times and temperatures on the preservation of the DHM functional components are presented. **Chapter 5** investigates the preservation of these compounds after non–thermal methods, i.e. HPP, UV–C and TUS, and discusses the DHM microbiological safety obtained with these methods. In **chapters 6**, **7** and **8**, the effects of thermal and non–thermal processing on specific DHM components and functions were studied; on the DHM insulin concentration in **chapter 6**, on the DHM antibodies against SARS–CoV–2 and on the HM's virus neutralization capacity in **chapter 7,** and on the DHM procoagulant activity in **chapter 8**. In **chapter 9**, all outcomes are discussed, and future perspectives are provided.

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Chapter 2

Testing the Effects of Processing on Donor Human Milk: Analytical Methods

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Abstract

Holder pasteurization is the current recommended method for donor human milk treatment. This method effectively eliminates most life–threatening contaminants in donor milk, but it also greatly reduces some of its biological properties. Consequently, there is a growing interest for developing novel processing methods that can ensure both microbial inactivation and a higher retention of the functional components of donor milk. Our aim was to offer a comprehensive overview of the analytical techniques available for the evaluation of such methods. To suggest an efficient workflow for the analysis of processed donor milk, a safety analytical panel as well as a nutritional value and functionality analytical panel are discussed, together with the principles, benefits, and drawbacks of the available techniques. Concluding on the suitability of a novel method requires a multifactorial approach which can be achieved by a combination of analytical targets and by using complementary assays to cross–validate the obtained results.

Keywords: Donor human milk; safety; functionality; nutritional value; milk bank; pasteurization

2.1. Introduction

Human milk (HM) is undoubtedly the optimal nutrition for all healthy neonates. Tailored to meet each infant's needs, it contains a large variety of nutrients and bioactive components that provide protection from infections and promote an infant's development and growth. According to the American Academy of Pediatrics (AAP), the World Health Organization (WHO), and the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), donor human milk (DHM) is considered the best alternative form of nutrition for vulnerable infants, in case of unavailability or insufficiency of mother's own milk. Policy reports and recommendations from the aforementioned official bodies specifically state that DHM should be distributed only through established human milk banks (HMBs) that have systems in place for proper quality control and are able to implement appropriate measures to ensure its safety [1, 2].

To ensure its microbial safety, DHM is pasteurized. The currently recommended DHM treatment is holder pasteurization (HoP) which requires heating DHM at 62.5 °C for 30 minutes followed by rapidly cooling it down to <10°C [3, 4]. Although this time–temperature combination effectively inactivates most life–threatening bacterial and viral contaminants, it negatively affects the concentration and activity of several bioactive components, such as hormones, growth factors, bioactive proteins, water soluble vitamins and enzymatic activities [5]. For this reason, improvements to the existing HoP procedure, either by optimizing thermal methodologies or applying non–thermal methodologies are increasingly being studied. For example, high–temperature short–time (HTST) pasteurization, high pressure processing (HPP), ultraviolet–C irradiation (UV–C) and (thermo–)ultrasonication are such methods [6].

Ensuring DHM safety and quality are the most crucial parameters when drawing conclusions about the efficacy of a new DHM processing method. To date, a number of different analytical methods are available to assess these parameters after processing of DHM. Consequently, due to the different analytical methods applied in the literature, comparing the effect of processing on DHM composition among studies may be challenging [6]. In fact, the

large variations frequently reported in studies investigating same DMH components after processing, are often attributed to the different analytical techniques used to assess such components [6–8]. As a step towards the harmonization of DHM analysis, this study aims to offer an overview of the safety, nutritional, and bioactive parameters that should be investigated in order to conclude on the efficacy of a processing method and to provide insights on the analytical procedures that can be used to study these parameters.

2.2. Safety

DHM is a highly complex biological fluid. A large number of harmless or even beneficial commensal bacteria have been isolated from DHM samples, most commonly of *staphylococci*, *streptococci*, *micrococci*, *lactobacilli*, and *enterococci* species [9]. However, whether it is DHM or own mother's milk, the presence of infectious pathogenic contaminants such as bacteria and viruses should be always considered, both due to exogenous and endogenous origins [10, 11].

Pathogenic and potentially pathogenic bacteria that could be considered as possible DHM contaminants, include a number of species that are frequently isolated from DHM samples or could be of concern in an HMB setting [4, 12]. Next to bacteria, also pathogenic viruses may exist in DHM. Cytomegalovirus (CMV) is a viral agent commonly found in DHM. Other viral contaminants relevant to DHM include the herpes simplex virus, hepatitis B and C, human immunodeficiency virus (HIV) and human T–lymphotrophic virus (HTLV) [13, 14]. To date, transmission of SARS–CoV–2 through human milk has not been proven [15].

2.2.1. DHM screening in a HMB

To eliminate this broad range of pathogenic micro–organisms, most HMBs pasteurize DHM. This method is effective at reducing both the bacterial and viral load in DHM to undetectable levels [4, 14]. In addition to pasteurization, HMBs follow strict screening criteria when recruiting a donor. Interviews, questionnaires and serological testing for hepatitis B or C, HIV, syphilis and in some cases CMV or HTLV are included in their screening process. HMBs additionally test DHM bacteriologically before and after pasteurization, to determine its acceptability. However, this is not a universally followed practice, as the timing and frequency of testing as well as microbiological acceptance criteria vary greatly among HMBs [12]. Most human milk banking guidelines recommend discarding DHM with total bacterial counts $>10^5$ colony forming units (CFU)/mL [4, 12, 16]. Stricter criteria may apply for microorganisms capable of producing thermostable enterotoxins, spores, and endotoxins (e.g. *Enterobacteriaceae*, *S. aureus*). Specifically for *Bacillus cereus*, a sporulating pathogen widely found in the environment, no consensus currently exists in the DHM microbiological screening among HMBs [17, 18]. Although cases of *B. cereus* infection in premature infants are extremely rare, neonatal sepsis due to *B. cereus* can be fatal [19]. However, the guidelines developed by the National Institute for Health and Care Excellence (NICE), suggest that through proper storage and handling, *B. cereus* DHM contaminations can be controlled [16]. In addition, milk banking guidelines indicate that post–processing, all pathogens should be absent [4, 12, 16].

2.2.2. Safety: Analytical panel

As the recipients of DHM are high–risk infants, all processing methods must meet the highest safety standards [6]. Therefore, the first step in the process of evaluating a method as suitable for DHM treatment, is to investigate the ability of this method to effectively eliminate life–threatening microbial contaminants in DHM. The techniques available for this purpose include an initial identification of the DHM native microbiota and a subsequent inoculation with a number of selected bacterial strains. A processing method can be further validated by additional viral inactivation experiments with a panel of DHM relevant viral agents. **Table 1** provides an overview of the analytical techniques available to assess the safety of a DHM processing method.

2.2.2.1. Bacteria identification and quantification

Before testing the effects of a DHM processing method against bacterial contaminants, the native microbiota of the DHM samples needs to be determined and quantified. For that purpose, both culture–dependent and culture–independent techniques have been used. Traditional bacterial culturing methods are routinely used, as they are less costly, detect only viable microorganisms and allow for further characterization of the isolates [20]. However, these methods are laborious, time consuming, highly dependent on the skills of the person performing the analysis and the equipment used [21]. The standard procedure includes the plating of DHM samples onto the appropriate media followed by an incubation period at optimum growth conditions. The bacterial loads can then be estimated quantitatively by colony counting (CFU/mL) [22]. Bacterial species can be further identified with colony morphology, Gram staining, and standard biochemical tests [21]. Molecular–diagnostic techniques such as polymerase chain reaction (PCR) sequencing techniques, genomic testing, and proteomics–based approaches like matrix–assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS) are widely used as well but are often not available in HMBs [23, 24]. These molecular–diagnostic techniques were initially developed to circumvent the limitations of the standard laboratory phenotyping methods [25]. Using ionization of the microbial cells, gene amplification, rRNA characterization, or sequencing of ribosomal genes, they quickly produce results and are known to be highly sensitive [21]. Distinction among species with comparable phenotypic characteristics is possible with those methods, as well as differentiation among strains of the same species [26]. However, the accuracy of molecular–diagnostic techniques relies on the sequencing quality and diversity. In addition, such methods are not able to detect viability of micro–organisms, but only their presence [20, 21, 27]. For further discrimination between viable and dead microbial cells, a selective membrane impermeable dye such as propidium monoazide is commonly used [28].

As the available techniques have both advantages and disadvantages, the selection of a method suitable for bacterial identification in DHM depends on the sensitivity required, equipment available and financial resources. Additionally, a combination of certain techniques (e.g. culturing methods and MALDI–TOF MS) may allow for even greater identification accuracy.

Table 1. Overview of the analytical methods available to evaluate the safety of a DHM processing method

DHM safety analytical panel	Methods	Techniques	Key points	Recommendations
Bacteria identification	Culture–dependent techniques	Traditional bacterial culturing methods *(plating onto appropriate media, incubation at optimum growth conditions, colony counting)* and identification based on colony morphology, gram staining, standard biochemical tests, microscopy	The native microbiota of the DHM samples should be determined prior to inoculation with the selected strains A combination of both culture–dependent and culture independent techniques will offer greater identification accuracy	DHM samples with no or very low native microbiota should be selected for inoculation with the appropriate bacterial strains
	Culture–independent techniques	Molecular–based applications *(PCR, genomic testing, MALDI–TOF MS)*		
Bacteria inactivation	Culture–dependent techniques	Inoculation of DHM samples with bacterial species that are frequently isolated from DHM samples or are of concern in a HMB setting (e.g. *Staphylococci, Enterobacteriaceae, Bacilli, Enterococci, Streptococci, Pseudomonas*). After processing, all samples are plated onto the appropriate nutrient media and incubated at optimum growth conditions. The reduction in bacterial counts is then determined after estimating the numbers of colonies on the plates	The selected strains must be clinically relevant to DHM and the inoculation level high enough to allow for the accurate calculation of a 5–$\log_{10}$ reduction The inactivation of biomarkers such as ALP, should be accompanied by bacterial inactivation experiments	A processing method is considered suitable for DHM treatment when a reduction in the total viable bacteria count of 5–$\log_{10}$ at minimum can be achieved
Viral inactivation	Seropositive mothers	DNA extraction and PCR amplification, neutralization assays, antibody detection assays and MS methods	The selected panel should include clinically relevant viruses for HM (e.g. *HIV, HTVL, cytomegalovirus*)	A processing method is considered suitable for DHM treatment when at least similar results to HoP can be achieved regarding viral inactivation
	Viral spiking experiments	PRA, TCID50, early antigen IF, cell culture toxicity, GFP indicator cells, RNA assay, RT activity, SEAP reporter, PBMC neutralization assay		

2.2.2.2. Bacterial inactivation

As required by the human milk banking guidelines, a method suitable for DHM treatment must be able to achieve a minimum of a 5–$\log_{10}$ reduction in the total bacterial load, which also includes bacterial species of clinical importance for DHM (e.g. *S. aureus*, *B. cereus*, *E. faecalis*, *S. pyogenes*, *S. agalactiae*, or *S. pneumoniae* and coliforms such as *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella spp*) [4, 9, 23, 29–31]. After the microbial contamination level of the DHM samples is determined as described in section 2.2.1, samples with low levels of microbes are then inoculated with a number of the aforementioned clinically relevant DHM bacterial strains, in a concentration of approximately 10^8 to 10^9 CFU/mL . This high level of inoculation is needed to enable the precise determination of the inactivation kinetics of the treatment method. Hence, studies evaluating the safety of a processing method by assessing the inactivation of the native DHM microbiota, should additionally include bacterial inactivation experiments in case the native DHM contamination is at a too low level for observing the minimum required 5–$\log_{10}$ reduction. When it comes to spore–forming species such as *Bacillus*, inoculation of DHM samples with both vegetative cells and spores will result in more accurate inactivation data [32]. Finally, the inactivation achieved is compared to the minimum required inactivation level [31].

2.2.2.3. Virus inactivation

HoP is regarded as effective against non–heat resistant viruses. To investigate the effect of DHM processing methods on inactivation of different viruses, most studies perform viral spiking experiments, using plaque reduction and endpoint titration assays (TCID50) or in some cases immunofluorescence, reverse–transcriptase enzymatic assays, or secreted embryonic alkaline phosphatase (SEAP) assays [33–37]. Another study included DHM from hepatitis B seropositive mothers that were submitted to DNA extraction and were further assessed with PCR assays [38]. Assessment of HIV viral infectivity before and after DHM heating using a peripheral blood mononuclear cell neutralization assay has also been reported [39].

2.2.2.4. Non–microbial biomarkers for safety

Alkaline phosphatase (ALP) is widely used in the dairy industry as an indicator of pasteurization efficiency. Complete inactivation of this enzyme is directly correlated with the inactivation of less heat–resistant pathogenic bacteria relevant to bovine milk (e.g. *Coxiella burnetti, Mycobacterium tuberculosis*) [40]. In HM, ALP may also serve as a beneficial enzyme [41]. The activity of this enzyme is usually assessed spectrophotometrically with the addition of p–nitrophenyl phosphate as a substrate, by calorimetric or fluorometric assays [11, 31, 40, 41]. However, more research is required in order to conclude on the sensitivity of this biomarker for DHM. The NICE guidelines state that a negative ALP test should be accompanied by bacteriological screening in order to ensure effective bacterial inactivation in DHM [16]. In addition, when DHM was subjected to UV–C, although sufficient microbiological inactivation was achieved, no loss of activity was documented [31]. Therefore, since ALP is heat–sensitive and may not be inactivated by non–thermal methods, a negative ALP test cannot serve as a replacement of microbiological testing when evaluating such methods.

2.3. Nutritional value

Mature HM has a macronutrient composition of approximately 0.9 to 1.2 g/dL protein, 3.2 to 3.6 g/dL fat, and 7.0 to 8.5 g/dL carbohydrates of which about 80% is lactose and 20% HM oligosaccharides (HMOs), and 0.2 g/dL minerals [42–44]. Due to the differences in lactational stage, maternal and environmental factors, the nutrient composition among different HM samples can be quite variable [42, 43]. To determine the effects of a DHM processing method on these macro– and micronutrients, it is therefore important to determine their levels and how they are affected by processing.

2.3.1. Macronutrient and micronutrient content

HM proteins can be classified into three major groups: caseins, serum proteins, and milk fat globule membrane (MFGM) proteins. HM contains three types of caseins, α–, β– and κ–casein, which together form colloidal particles in milk known as casein micelles [45, 46]. The serum protein fraction includes many proteins, with α–lactalbumin and lactoferrin among the most abundant. The serum /casein ratio of mature HM is approximately 60/40. MFGM is comprised by a group of various proteins, which include mucin and lactadherin. Non–protein nitrogen compounds, such as urea, uric acid, creatinine, free amino acids, represent around 25% of the total nitrogen in mature HM [43]. Nutritionally, HM proteins serve as amino acid sources. Since the amino acid composition of serum proteins and caseins differs, and their ratio differs throughout lactation, the HM amino acid profile varies as well [47].

HM lipids are known to be highly variable. Factors such as the lactation stage and maternal diet have been shown to influence their composition [48]. HM lipids are considered as the main energy source, accounting for approximately 50% of total HM energy content [45, 49]. Triacylglycerides (TAGs) represent 98% of the HM lipids while the remaining fraction consists of diacylglycerides, monoacylglycerides, phospholipids, cholesterol, and free fatty acids [45]. Fatty acids that are available in high concentrations in HM include linoleic, palmitic, and oleic acid [43, 50]. In mature HM, 32–52% of the fatty acids are saturated, 30–50% are cis–monounsaturated, 2.5–13.8% are trans–monounsaturated, while the rest are polyunsaturated fatty acids (PUFA) [42].

Lactose and HMOs are the most abundant carbohydrates in HM. Lactose is the main disaccharide in HM, and its concentration varies the least among the HM nutrients. However, lactose concentration tends to be higher in the milk of mothers who produce higher amounts [51]. In mature HM, lactose concentration is 67–78 g/L [43]. HMOs concentration in colostrum is 20.9 g/L on average and in mature HM, 12.9 g/L [44, 45]. HMOs are considered nonnutritive to the infant, but with great functional properties [43].

Vitamin content usually depends on maternal diet and her general nutritional status. However, other physiological or environmental factors may also influence their concentration. In case of vitamin insufficiency, supplementation is commonly recommended [42]. Apart from vitamin D, which can be found in the serum fraction, the other fat–soluble vitamins are associated with the MFGM. HM is low in vitamin K, while the concentration of vitamins A, C, B1, B2, B3, B6, B12 and D mostly depends on maternal diet, supplement use and body stores [42, 43]. Mineral concentrations change during lactation, with higher values documented in

colostrum than mature HM. Aside from selenium, fluorine, and iodine, HM mineral concentration is generally not dependent on maternal diet [42].

2.3.2. Nutritional value: Analytical panel

After establishing the safety of DHM, its nutritional quality and bioactivity should be assessed, to draw conclusions regarding the suitability of a method for DHM processing. It has been documented that a large number of key nutritional factors remains unaffected after HoP [4, 8]. Lactose, fatty acids, the majority of minerals and vitamins A, D, and E are included in those heat–stable factors [4, 52]. The contrasting results among different studies on the effect of HoP on other nutrients, such as lipids, may be explained by the different analytical techniques used for their measurement [8]. Therefore, since processed DHM should remain nutritionally sufficient for infant feeding, assessing the preservation of its nutritional value using a number of analytical methodologies, is of great significance. In addition, accurately determining the concentration of several DHM components is an important step when implementing a novel method, as e.g. in the case of UV–C irradiation, the appropriate dosage for sufficient bacteria inactivation was found to depend on the DHM total solids concentration [31]. **Table 2** offers an overview of the analytical techniques available to evaluate the effect of a processing method on DHM nutritional value and the minimum requirements for the results of these analyses.

2.3.2.1. Macronutrient content

To measure the macronutrient content in HM, HMBs in general use commercially available human milk analyzers (HMAs). Most of these devices can quantitatively measure the concentration of fat (total lipid–soluble fraction), protein, carbohydrates (lactose and oligosaccharides), total solids, and energy in HM, using mid–infrared (MIR), Fourier transform infrared (FT–IR) or near–infrared (NIR) spectroscopy [53–55]. These methods are based on the interaction between specific infrared irradiation wavelengths with various chemical groups of HM components [53]. The technology employed by these devices allows for rapid HM analysis, using only a small amount of HM [56]. However, measurements of the total carbohydrate content have been reported to be less accurate compared to other nutrients, among the available devices [54]. Especially for lactose quantification, MIR spectroscopy may give poor results, as the presence of oligosaccharides can affect the measurement [57]. In addition, studies measuring total lipid content with HMAs found a significant decrease after HoP, which was not documented in studies using reference laboratory methods. This may be further explained by the fact that infrared analyzers do not measure fat content directly [8]. As a general guideline, the calibration, validation, and quality assurance of HMAs must be ensured, in order to obtain accurate and precise measurements [58].

Reference laboratory analytical methods are considered as the gold standard [59]. For total protein determination, the Kjeldahl or Dumas methods are routinely used. These methods are based on total nitrogen estimation [59, 60]. Other methods include the biuret assay, the Lowry–Peterson assay, the Bio–Rad Coomassie Blue assay, and bicinchoninic acid (BCA) protein assay, which are all based on the spectrophotometric analysis of a colorimetric reaction [60, 61]. Studies comparing the protein values obtained by these methods to the values measured with Kjeldahl concluded that the BCA assay showed the greatest precision [62, 63].

This assay is most often used nowadays, due to its sensitivity and simplicity but an overestimation of the protein concentration measured has been reported as well [61]. Since this is a colorimetric assay, HM samples must be completed defatted, in order to avoid the milk fat light–scattering effects. Similarly, sample preparation, incubation time, and temperature can greatly affect its accuracy, therefore every step needs to be well–controlled [63, 64].

To determine HM total lipid content, solvent extraction followed by gravimetry is the most widely used method. The Folch, Röse–Gottlieb and its modified methods (e.g., Mojonnier method) follow this principle. The fatty acid composition can be analyzed by using high resolution gas chromatography (HR–GC), after preparing the fatty acid methyl esters (FAME) of milk fat by transesterification [57, 65, 66].

High performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC–PAD) is regarded as a selective and sensitive method for lactose quantification [63]. Enzymatic assays that colorimetrically quantify glucose or galactose after lactose hydrolyzation, are also commonly used. However, high performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) methods are considered superior to the enzymatic assays.

In summary, for practical reasons HMAs are most suitable for suitable for DHM analysis in a HMB setting [67], even though significant differences in the DHM macronutrient concentration between HMA and the reference laboratory methods have been described [8, 68]. Therefore, for the analysis of individual DHM components, reference laboratory methods may be preferred, in order to obtain the most accurate results.

2.3.2.2. Micronutrient content

Many methods have been used for the micronutrient analysis of HM. For the determination of B–vitamins, HPLC separation followed by fluorescence detection, ultraperformance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS), nuclear magnetic resonance (NMR) spectroscopy and microbiological assays are most often used [69]. UPLC–MS/MS, specifically, is considered to have offered improved resolution and analytical sensitivity in such analyses [57]. HPLC with fluorescence, ultraviolet (UV), or electrochemical detection are the most recent methods used for vitamin C quantitation in HM. HPLC combined with fluorescence or UV detection and liquid chromatography–tandem mass spectrometry (LC–MS/MS) approaches are considered as suitable methods for vitamin A and vitamin E determination of HM. With regards to vitamin D analysis, competitive protein–binding assays, HPLC or LC–MS/MS methods are applied [69].

Several studies indicate that fat–soluble vitamins may not be affected by HoP while water–soluble vitamins (especially vitamin C) may significantly be reduced. For some B vitamins, contradictory results of HoP have been reported. These variations could be attributed to the different analytical methods used, study sampling and vitamin instability [8].

Atomic absorption spectroscopy (AAS), inductively coupled plasma–atomic emission spectroscopy (ICP–AES) and inductively coupled plasma–mass spectrometry (ICP–MS) are the methodologic approaches preferred for analyzing mineral concentrations in HM [69]. These methods have been shown to be superior to the colorimetric approaches previously used for mineral analysis [69]

Table 2. Overview of the analytical methods available to evaluate the effect of a processing method on DHM nutritional value.

DHM nutritional value analytical panel	Methods	Techniques	Key points	Recommendations
Macronutrient & micronutrient composition	Human milk analyzers	Measurements of energy, fat, protein, lactose, oligosaccharides, and total solids using MIR, FT–IR or NIR spectroscopy	Especially with regards to the estimation of the DHM protein and lipid content, significant differences have been reported between human milk analyzers and laboratory–based methods Laboratory–based methods are the gold standard for such analysis With the exception of several vitamins, the nutrition value of DHM is expected to be relatively unaffected by the processing methods	The nutritional value of DHM should be retained after processing with a novel method, to at least the same level as HoP
	Laboratory–based methods	Total protein determination by measuring total N (e.g. Kjeldahl, Dumas), or by spectrophotometrically analysing a colorimetric reaction (Biuret, Lowry–Peterson, Bio–Rad Coomassie Blue and BCA assays) Total lipid content determination by solvent extraction followed by gravimetry (e.g. Folch, Röse–Gottlieb, Mojonnier methods) and fatty acid composition determination (e.g. HR–GC with FAME) Lactose quantification (e.g. HPAEC–PAD, HPLC, LC–MS, enzymatic assays with colorimetric detection,) Determination of vitamins and minerals (e.g. HPLC with fluorescence, UV or electrochemical detection, UPLC–MS/MS, LC–MS/MS, AAS, ICP–AES, ICP–MS)		

2.4. Functionality

HM contains a large number of immunological factors, cells, antimicrobial peptides, hormones, growth factors and probiotics that have a profound role in infant development and health [42]. As a significant reduction in the concentration of several HM bioactive components has been demonstrated after HoP, preserving their biological activity during HM processing remains of critical importance [6, 8, 43]. Since current evidence suggests that HMOs are not affected by HoP [8], the analytical methods available to assess these components are not further discussed in the present study.

2.4.1. Bioactive HM proteins

Alpha–lactalbumin, lactoferrin (LTF), lysozyme (LYZ), secretory immunoglobulin A (sIgA) and serum albumin are the most abundant proteins in the serum fraction [42]. The most abundant immunoglobulins in HM are sIgA, followed by sIgG and sIgM [42]. As the largest antibody system in HM, sIgA acts directly at the mucosal surface, thus protecting the infant form invasive pathogens. It inhibits the binding of microbial agents to epithelial cells, enhances phagocytosis, and further regulates local immunity [42]. Hormones and growth factors such as insulin, leptin, adiponectin, ghrelin, erythropoietin, calcitonin, somatostatin, and epidermal, neuronal, vascular endothelial and insulin–like growth factors are also present in HM. They are involved in the maturation and protection of the gastrointestinal tract, in metabolic regulation as well as in promoting immune tolerance [42, 43]. In addition, a great number of peptides that occur naturally in HM, formed by enzymes such as plasmin, cathepsin and elastase, are known to have a range of antimicrobial and immunomodulatory functions [70].

LTF represents approximately 15% to 20% of the total HM protein content and is considered as one of the principal antimicrobial HM components [71]. It is an iron binding glycoprotein with known bacteriostatic activity against a variety of pathogenic microorganisms. LTF can inhibit the growth of iron–dependent pathogens, e.g. *E. coli*, by limiting the availability of free iron but also by increasing the permeability of the bacterial cell membrane. LTF exerts additional inhibitory effects against viruses, by binding to surface proteoglycans or through other interactions with viral envelope proteins [72].

LYZ is another major antimicrobial protein in HM. This protein is present in high concentrations (0.3–0.5 g/l) and it remains quite steady throughout lactation [42]. When acting alone, LYZ is capable of killing gram–positive bacteria by lysing the proteoglycan matrix located in their cell wall [71]. Against gram–negative bacteria, LYZ acts together with LTF; after the latter binds to their outer cell membrane, LYZ is able to access and break down their inner proteoglycan membrane, causing their elimination [42, 47].

Bile salt–stimulated lipase (BSSL) is a multifunctional enzyme, also present in high quantities in HM. It catalyzes triglyceride digestion, thus improving lipid and lipid–soluble vitamins absorption and enhancing infant metabolism [73]. Due to the fact that the activities of this enzyme are completely lost after HoP, the fat absorption and the growth rate of preterm infants fed with pasteurized DHM was lower compared to infants fed raw own mother's milk [31, 74].

Another HM bioactive protein is lactoperoxidase (LPO). In the presence of hydrogen peroxide, LPO can convert thiocyanate to hypothiocyanate, which is considered as a growth inhibitor of both gram–positive and gram–negative bacteria [47].

2.4.2. Lipids

Apart from providing energy, the lipid fraction in HM contains a number of bioactive components such as cholesterol, glycerophospholipids, sphingolipids, and glycolipids, with anti–infectious, antioxidative and anti–inflammatory activities [43, 45]. These compounds are implicated in a wide range of cellular functions, such as infant growth, neurobehavioral and brain development [45, 75]. In most studies, HoP did not reduce the total DHM lipid content or affect the FA profile [8]. However, intense non–thermal processing methods may lead to formations of lipid oxidation [3]. For example, HPP at 600 MPa for durations longer than 3 minutes was shown to result in the formation of compounds associated with lipid oxidation, such as aldehydes [76, 77]. Similarly, volatile compounds from lipid oxidation have been detected after extended ultrasonication of bovine milk [78, 79], whereas for UV–C, some studies did report the occurrence of oxidation, while others didn't [31, 80–82] which may depend on the treatment intensity.

2.4.3. Functionality: Analytical panel

HoP has a negative effect on the concentration and functionality of several DHM bioactive components, with proteins being most significantly affected [8]. Therefore, after ensuring DHM safety as described in section 2, a method suitable for DHM processing should be able to preserve the DHM bioactive components and their function better than HoP. Currently, several methods are available to perform such testing. Both untargeted and targeted techniques can be used for the analysis of the different DHM functional components. Typically, untargeted approaches can produce both qualitative and semi–quantitative data, while targeted approaches are used to obtain quantitative data [83, 84]. **Table 3** represents an overview of the analytical methods available to evaluate the effect of a processing method on DHM functionality.

2.4.3.1. Targeted protein determination

For a targeted identification of proteins like sIgA, LTF and LYZ, generally available enzyme–linked immunosorbent assays (ELISAs) are mostly used. The antibodies used in such assays recognize the tertiary structures of those proteins and therefore can differentiate between their native and denatured forms. Immunostaining, MS, and radial immunodiffusion assays (RIAs) have been used as well [8]. Cytokines, hormones, and growth factors are mostly characterized by using specific ELISA or RIAs [8].

ELISA is generally regarded as the gold standard of immunoassays. It's a sensitive, rapid, and cost–effective method, designed for the selective detection and quantification of targeted substances (e.g. antigens, antibodies, proteins, peptides, glycoproteins, and hormones) [85]. These substances can be detected by a colorimetric reaction following the binding of an antigen to a specific enzyme–labeled antibody [85, 86]. The limitations of these assays include cross–reactivity, nonspecific antibody recognition, the additional costs of developing well–characterized antibodies, and the poor repeatability of measurements that has been reported in some studies [86–89].

The first immunoassay ever developed was a RIA, which uses a radiolabeled antigen that competes with the antigen of interest in the sample, for binding to a specific antibody. The radioactivity of the antigen–antibody complex is then measured and used to calculate the antigen concentration [90]. Since radiolabeled antigens are used, interference risks from the sample itself are limited [90]. In addition, they are considered as quite fast and have a high sensitivity. However, the use of special facilities and radioactive materials is required. Such materials may be associated with health risks, have short shelf–life, and their disposal can be also an issue [90–92].

Gel electrophoresis, including 1–dimensional (1D) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) or 2–dimensional (2D) PAGE, combined with protein staining, can be used to separate proteins based on their molecular size and further investigate the changes induced by the DHM processing methods (e.g. denaturation, aggregation or the degree of carbonylation) on the protein pattern [93–95]. Such techniques may lack the quantitative results that an ELISA can offer, but the qualitative information they provide can be used to complete the analytical panel of the different methods.

LC/MRM–MS is a mass spectrometric technique that is considered as a highly specific and selective technique for the quantification of various compounds in complex mixtures. Targeted LC/MRM–MS techniques have been used for the quantification of certain proteins in bovine milk, such as β–lactoglobulin, individual caseins, and specific MFGM proteins [96, 97]. The principle of this measurement relies on the targeted fragmentation of specific peptides from the protein of interest and of selected signature peptides, and the subsequent monitoring of the ions formed that are specific to those peptides, which can then be used for their quantification. [97]. Despite the high selectivity of this method, interferences such as the co–elution of peptides with ion masses similar to the specific peptides may limit its sensitivity [98]. Therefore, ensuring appropriate fraction preparation of complex mixtures is of particular importance [99].

As protein–protein interactions and synergies have become more relevant, current research focuses more and more on the complete protein profile analysis. Therefore, untargeted techniques that allow proteome quantification and characterization of protein interactions and modifications are being increasingly used.

A detailed characterisation of the HM protein fraction, including the detection of less abundant proteins and their degradation products, can be achieved by applying –omics approaches such as proteomics, peptidomics, and glycoproteomics [100]. These approaches are gaining more ground recently, due to the amount of information that can be acquired [8]. In fact, LC–MS/MS based shotgun proteomics is considered as a robust method for quantifying the effects of the different processing methods on DHM protein preservation [101, 102].

For studying the HM proteome, HM is usually fractionated into its major protein classes (caseins, serum and MFGM proteins). The majority of the bioactive proteins in HM can be found in the serum fraction, also called whey fraction, which can be isolated by ultracentrifugation of HM that has been previously skimmed [103]. To detect possible protein damage as an effect of the different DHM processing methods, an additional step of acidification (pH 4.6) should precede ultracentrifugation in order to separate the denatured and aggregated serum proteins from the native HM serum proteins [102].

Table 3. Overview of the analytical methods available to evaluate the effect of a processing method on DHM functionality.

DHM functionality analytical panel	Methods	Principles and techniques	Key points
Bioactive proteins	Untargeted protein profiling	Detailed characterisation of the DHM proteome, including less abundant proteins with labelled and label–free proteomics, glycoproteomics, peptidomics (e.g. LC–MS/MS with FASP)	Investigating the retention of the DHM bioactive proteins is the second most important step when evaluating a processing method HoP significantly reduces the concentration of various DHM bioactive components, such as immunoglobulins, lactoferrin, lysozyme, a number of cytokines, hormones and growth factors. In addition, HoP causes the complete elimination of BSSL activity. A higher retention of the functional DHM that are negatively affected by HoP should be obtained with the novel methods Total protein quantification and protein specific quantification methods should be combined by functionality assays to facilitate the accurate evaluation of the effects of a DHM processing method on its quality (e.g. ELISA assays or MS–based techniques combined with antibacterial activity assays to assess functionality retention of DHM bioactive proteins or combined with complimentary neutralization assays for the detection of specific DHM antibody responses)
	Targeted protein determination	Detection of a targeted antigen using a specific enzyme–labelled antibody (e.g. ELISA, RIA, electrochemiluminescence immunoassays) Measurement of the ions specific to the peptides that correspond to the protein of interest through a triple quadrupole MS (LC–MRM/MS)	
	Functionality assays	Proteins can be separated based on their molecular size while by using SDS–PAGE separation techniques, while obtaining qualitative information on the changes induced by the processing in the protein pattern Enzymatic activity assays, antibacterial activity assays, assays that determine the neutralization capacity of specific antibodies	
Lipidome	Targeted and untargeted approaches	GS, SFC, MRM, MS–based techniques (LC–MS, UPLC–MS/MS, FTICR, Orbitrap, QTOF–MS)	DHM lipids are considered as essential for infant growth, and brain development. Targeted approaches are able to produce data less complex and are considered as the preferred approach in the quantification of low abundance lipids. Untargeted approaches offer a broad analysis of the DHM lipidome, although often used semi–quantitatively. The majority of the available studies found no effect of HoP on the DHM lipid content and total fatty acid profile, while only a few reported a significant decrease.

2.4.3.2. Untargeted protein profiling

For cleanup and digestion of the protein, filter–aided sample preparation (FASP) is a highly applicable technique that is broadly used in proteomic analyses. The high quality of the peptides generated with FASP is a great advantage over other sample preparation methods [104]. Both labelled and label–free proteomics have been combined with FASP and subsequently LC–MS/MS analyses to accurately determine the DHM proteome [103, 105].

2.4.3.3. Functionality assays

HoP has been shown to only partially preserve the immunological proteins in DHM and to significantly reduce the concentration of several other bioactive compounds [8, 106]. To determine the effects of a processing method on DHM functionality, after estimating the concentration of the bioactive components present in DHM, the retention of their bioactivity should be defined. For that objective, assays that evaluate the extend of bacterial proliferation after treatment, assays that determine the virus neutralization capacity of specific antibodies and activity assays utilizing the spectrophotometric detection of a colorimetric reaction, are most broadly used.

HM contains a large number of bioactive enzymes and their activities are often detected by allowing them to convert a synthetic substrate into a product that can be measured spectrophotometrically. BSSL, LPO and ALP activities are usually determined in such way [8, 106]. BSSL activity can be additionally measured with commercially available kits, but for LPO this is quite challenging, due to the low concentration of this enzyme in HM [8, 31, 107]. Lastly, for the detection of specific HM antibody responses, mostly ELISA assays or MS–based methods with complimentary neutralization assays are used [108, 109].

The functionality of HM is often assessed by the retention rate of its bacteriostatic properties, which are considered as the result of the activities of the whole range of antibacterial HM proteins. These assays include the inoculation of untreated and treated HM samples with certain gram–negative and gram–positive bacteria (e.g. *E.coli* and *S. aureus*) and the subsequent evaluation of their growth rate in those samples [106, 110, 111]. Untreated HM samples are used as a reference of the maximum bacteriostatic activity exerted by the bioactive HM proteins, since they are present in their native forms. Higher bacterial growth rate than the one observed in untreated HM samples is thus correlated with reduced antimicrobial activity [106]. Specifically for the determination of LYZ activity after DHM treatment, most studies use a *Micrococcus lysodeikticus*–based turbidimetric assay. This assay measures the reduction in the cell suspension turbidity as a result of *M. lysodeikticus* cell lysis. However, the sensitivity of this method could be an issue in the accurate determination of LYZ activity in HM [8].

However, determining the exact proportion of the bioactivity of specific proteins using such functional assays, without considering the great number of immune components in HM with known antimicrobial activity and the synergistic effects observed, remains challenging. On the other hand, these assays are able to demonstrate whether the antimicrobial activity of HM is overall influenced by the different processing methods [106, 112]. Combining such assays with methods that allow a detailed characterization of the bioactive HM components (e.g. proteomics) is considered as an efficient workflow for HM analysis [106, 112].

2.4.3.4. Lipidomics

Lipidomics approaches, both targeted and untargeted, may offer a comprehensive and quantitative description of the HM lipidome profile. Such approaches are widely applied in biological research, but their application in HM is quite recent [113]. GC, supercritical fluid chromatography (SFC), MS–based and NMR spectroscopic techniques are the major techniques available for the qualitative analysis of DHM lipids. GC and LC–MS are commonly used for FA quantification; the latter may additionally offer analysis of intact lipids, while GC may lack mass selectivity [113]. SFC is a separation method similar to LC, with lower cost and higher resolution, but very few data are available regarding the application of this method in HM. MRM is often employed for studying targeted lipids or lipid classes, while untargeted lipidomics is usually performed by MS–based platforms, such as UPLC–MS/MS, Orbitrap, Fourier transform ion cyclotron resonance (FT–ICR) and quadrupole–time–of–flight (QTOF) [114] In general, targeted approaches generate less complex data, and are usually of preference when the objective is the quantification of low abundant lipids, such as branched–chain fatty acids (BCFAs) [83, 115]. In contrast with targeted lipidomics, untargeted approaches provide a detailed analysis of the lipidome, but they are often used semi–quantitatively [83, 113]. For a broad lipidomic analysis, the complexity of the HM matrix, the variability as well as the hydrophobicity of the HM lipids may be considered as potential challenges [113]. The application of lipidomics in HM could be further improved with the development of a wider lipidome database and a more standardized analytical workflow [113].

Finally, the extent of lipid oxidation in HM as an effect of processing can be assessed by the spectrophotometric quantification of specific oxidation products, such as conjugated dienes, lipid peroxides and thiobarbituric acid reactive substances [116]. HPLC with fluorescence detection is also commonly used for the determination of malondialdehyde, which is a lipid peroxidation end–product [81]. Hexanal, a major volatile aldehyde formed as a secondary HM lipid peroxidation product, is another reliable lipid oxidation marker, which can be detected by solid phase micro extraction GC–MS [81, 117]. Lastly, acid degree values analysis together with sensory evaluation are often used as an indicator of off–flavors in milk [82].

2.4.3.5. Future research on the analysis of DHM immune components

An increasing number of studies aim to investigate the relationship between the cellular HM components and the innate immune system [118]. Maternal immune system components, such as leukocytes, have been detected in HM, while the discovery of HM–derived stem cells paved the way for additional research on the possible therapeutic use of HM and its involvement in infant development [119, 120]. The analytical methods available for their characterization may offer an overview on cell phenotype and expression, concentration, and viability. Flow cytometry, immunofluorescence labeling and quantitative reverse transcription polymerase chain reaction (qRT–PCR) are the methods most commonly used. The various flow cytometry–based techniques available are considered to be high–throughput tools for HM leukocyte identification. HM cell extraction and subsequent culturing for further analysis has been also reported [120, 121].

Functionality assays using HT–29 and Caco–2 reporter cell lines for the activation of regulatory factors, such as the nuclear factor kappa B (NF–kB), have also been used for the *in vitro* evaluation of certain HM modulatory effects [122, 123]. For example, HoP was found to significantly reduce the NF–kB modulatory capacity of HM induced by tumor necrosis factor–α, using an HT–29 cell line [122].

The role of HM exosomes and the microRNAs (miRNAs) they contain, on the infant's immune function and cellular development, is another emergent topic [124]. Exosomes can transfer miRNAs to immune cells, which is considered as a mechanism of genetic exchange between cells, which may affect a number of physiological functions [125]. Several methods are currently available for exosome purification, with differential centrifugation, as well as solution sedimentation and low–speed centrifugation being most commonly used [126]. Some limitations regarding the quality of the exosomes isolated are reported for differential centrifugion, while both techniques are often considered costly and time–consuming. Detailed characterization of the exosome proteome has been achieved by LC/MS–based [126]. To further study the miRNAs in HM, miRNA isolation, library preparation, and subsequent sequencing is usually performed [125, 127]. Limited data are currently available on the impact of processing on those bioactive compounds. One study comparing the impact of HoP and HPP on the HM miRNA content, reported a significant degradation in HoP samples while HPP was found to be a less damaging treatment [125].

Lastly, there's a large number of studies available documenting processing–induced damage to bovine milk [101, 112, 128]. These findings may lead to a better understanding of milk components in general and could be additionally translated to HM research. Therefore, future possibilities for HM analysis, including an understanding of its functionality, may therefore come from bovine milk research, which could thereby facilitate an acceleration in scientific and technological applications for HM research.

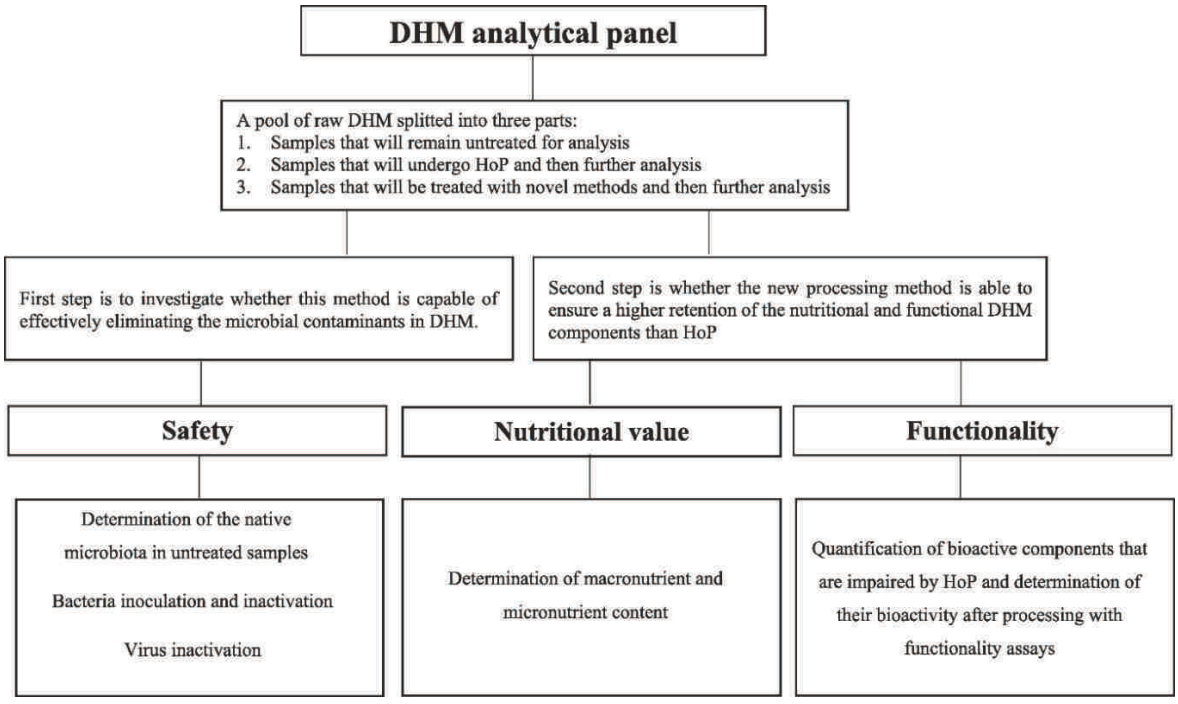

Figure 1. A summarized overvierw of a suggested workflow for evaluating the suitability of a novel method for DHM processing

2.5. Conclusion

As the detrimental effects of HoP on several HM components have been well–described, the interest in novel DHM processing methods has grown rapidly. For a processing method to be regarded as a suitable, it should ensure as prerequisite microbial inactivation at least as effectively as HoP while at the same time, higher retention rates of the DHM nutritional and bioactive elements should be achieved. A detailed overview of the available analytical techniques that can be used to evaluate these processing methods are presented in this study. Pointing towards an efficient DHM analytical workflow, a safety panel is introduced as the primary analytical step, followed by the assessment of the preservation of its nutritional value and functionality **Figure 1** presents a summarized suggested workflow for concluding on the suitability of a novel method for DHM processing. Whether DHM quality is affected by a treatment and to what extent, can be determined by combining targeted or untargeted analytical techniques with functionality assays. In addition, by using complementary assays, the variety and variability of certain DHM components after treatment can be further validated. Future studies evaluating DHM processing methods could benefit from adopting a more standardized analytical workflow, including all three analytical panels.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not–for–profit sectors.

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Chapter 3

"Donor milk banking: Improving the future". A survey on the operation of the European donor human milk banks

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Abstract

Provision of donor human milk is handled by established human milk banks that implement all required measures to ensure its safety and quality. Detailed human milk banking guidelines on a European level are currently lacking, while the information available on the actual practices followed by the European human milk banks, remains limited. The aim of this study was to collect detailed data on the actual milk banking practices across Europe with particular emphasis on the practices affecting the safety and quality of donor human milk.

A web–based questionnaire was developed by the European Milk Bank Association (EMBA) Survey Group, for distribution to the European human milk banks. The questionnaire included 35 questions covering every step from donor recruitment to provision of donor human milk to each recipient. To assess the variation in practices, all responses were then analyzed for each country individually and for all human milk banks together.

A total of 123 human milk banks completed the questionnaire, representing 85% of the European countries that have a milk bank. Both inter– and intra–country variation was documented for most milk banking practices. The highest variability was observed in pasteurization practices, storage and milk screening, both pre– and post–pasteurization.

We show that there is a wide variability in milk banking practices across Europe, including practices that could further improve the efficacy of donor human milk banking. The findings of this study could serve as a tool for a global discussion on the efficacy and development of additional evidence–based guidelines that could further improve those practices.

3.1. Introduction

Human milk banks (HMBs) select, collect, screen, store, process and distribute donor human milk (DHM) that is intended for high–risk infants [1–3]. Since operational safety and quality assurance is considered as a key priority for all HMBs, each practice should be well monitored, and a quality control system should be implemented [1, 4, 5]. Donor recruitment and screening, milk expression, handling and storage (conditions, temperature, duration) both at donors' homes and in HMBs, transportation to the milk bank (if applicable), bacteriological testing, quality control, pooling, thawing and pasteurization of DHM are included in those practices.

According to the European Milk Bank Association (EMBA), there are currently 248 HMBs located in 26 European countries [6]. Most HMBs operate based on locally implemented standards, nationally or internationally published guidelines. Guidelines published or translated in English are available from the UK, France, Italy, Spain and Sweden. Other countries with nationally recognized guidelines include Germany, Austria, Norway, Slovakia, and Switzerland [1]. HMBs in Poland and Estonia follow internal procedures of conduct that are not subjected to legislation nor are they monitored on a national level. Currently, DHM is not under EU legislation. In Austria, the existing recommendations are legally binding and only in France and Italy federal authorities are closely regulating DHM services [7, 8]. Differences among existing guidelines are mainly due to variations in practices, organization and regulation of HMBs throughout Europe. Those differences include DHM legal classification, location and

distribution area of each HMB, and lack of evidence for standardization of some operational points [1, 2 ,7].

As no European–wide published guidelines were available, EMBA's Guideline Working Group was convened in 2015 to undertake this task. Group members from 13 countries (Austria, France, Germany, Italy, Norway, Poland, Portugal, Serbia, Slovenia, Slovakia, Spain, Switzerland, and the UK) completed a detailed survey on the practices followed by their national HMBs. The group investigated whether a consensus on practices was apparent and whether published evidence was available to support recommendations. The EMBA Recommendations for the establishment and operation of human milk banks in Europe became available in 2019 [1]. Notwithstanding the foregoing, and studies on actual procedures in some European countries, a pan–European overview of milk banking practices is lacking and may differ from these recommendations, even among HMBs within individual countries. The aim of the present study was to collect detailed data on the human milk banking practices in Europe, with particular focus on human milk donation, storage, handling, screening and treatment. The outcomes of this study will be used to further strengthen human milk banking guidelines and recommendations.

3.2. Materials and Methods

The EMBA Survey Working Group developed a structured web–based questionnaire on milk banking practices, to subsequently distribute to all HMBs that were actively operating in Europe at that time (n=226, April 2019, EMBA [6]). A list with names and locations of 194 active HMBs in 26 European countries was created, with the joint effort of EMBA and the NGO PATH. Email addresses of 152 HMBs were initially available. The list was then updated and a total number of 215 HMBs with available contact details was finally obtained. Due to a lack of contact details, HMBs in Slovakia and Hungary (n = 11) could not be included in the final list. National coordinators from all 26 countries were appointed, to assist with survey distribution and completion. Their role included updating the number of active HMBs in their own countries, encouraging participation of those HMBs and lastly, minimizing linguistic barriers by offering a native language version of the questionnaire when required (**Figure 1**).

A general data protection regulation compliant online platform (SurveyMonkey, Portland, USA) was used to facilitate data collection. The selected questions (n=35) targeted the most critical aspects of the standardized procedures followed in HMBs: donor screening, handling, storage, processing, and microbiological testing of DHM. HMBs had to answer all questions, with the exception of HMBs that do not pasteurize DHM. In that case, HMBs could skip the group of questions regarding pasteurization (n=7). The Bioethics Committee at Warsaw Medical University reviewed the current study and declared no objection on its conduction (KB/O/23/2021).

A survey invitation email with a web–link to the questionnaire was first sent in April 2019, along with a cover letter explaining the purpose of the study. The letter additionally included detailed information on confidentiality, survey conduction, and contact details of the head of the working group, in case further explanation was needed. Reminders were sent to all participating HMBs in July and August 2019. Next, the authors further contacted all HMBs

with incomplete or unclear responses as well as all HMBs with contact details received after July 2019. The survey link remained active until November 2019.

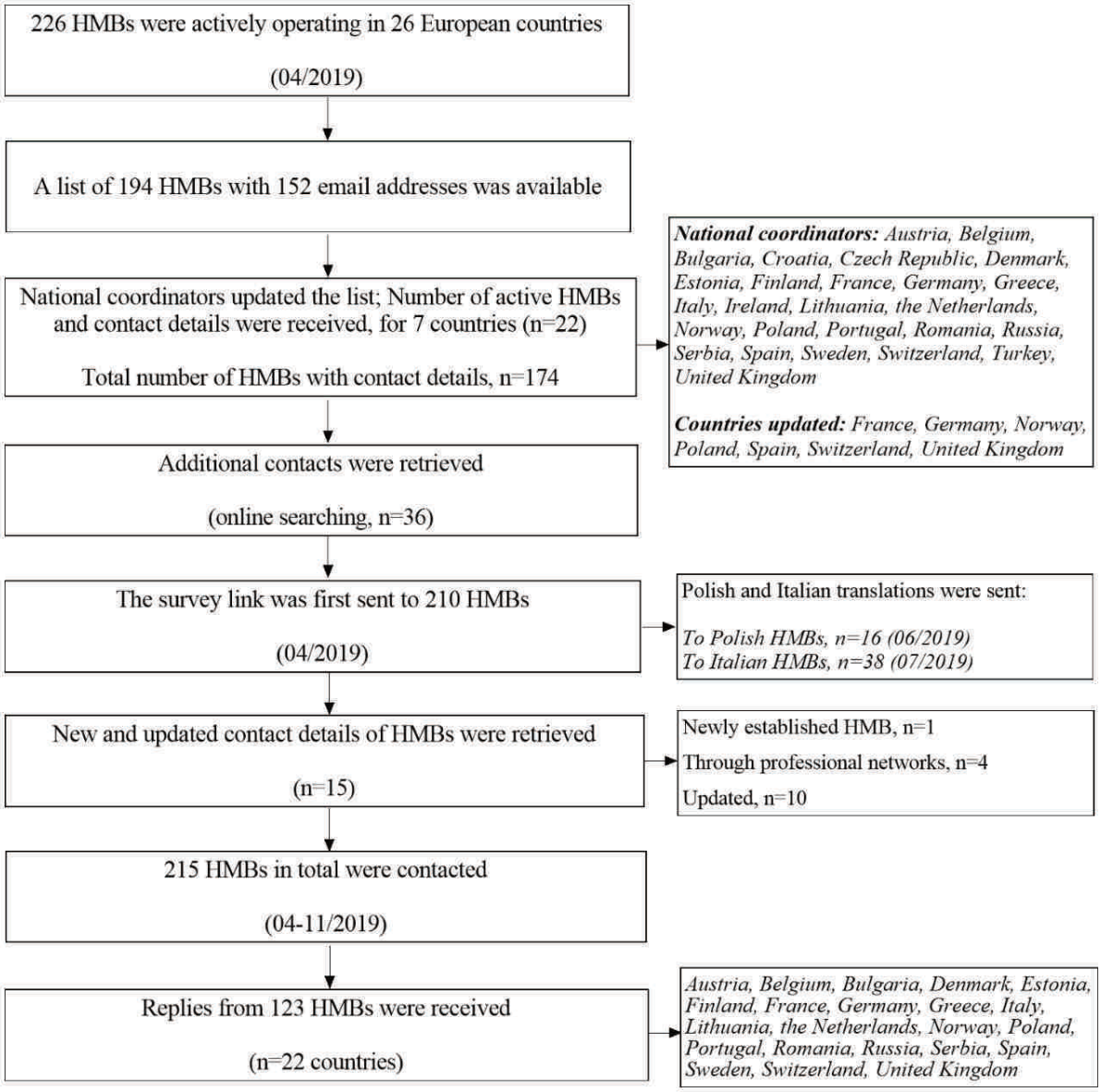

Figure 1. Schematic chart indicating participant flow

Once the survey was completed, all responses were screened and categorized (per country, per question, per HMB, and as a whole) using Microsoft Excel (2010). GraphPad Prism software 8.0 (GraphPad Inc., La Jolla, CA) was then used for data analysis and visualization. To assess the variation in milk banking practices, adherence to guidelines and extent of milk banking activity, responses for each question were analyzed both for each country separately and for all HMBs together. All calculated percentages were rounded up to the nearest integer. The questionnaire and the list with the responses received per country are available as **Supporting Information S1** and **S2**.

3.3. Results

A total of 123 replies (response rate=57%) from 22 out of the 26 European countries (85%) were received (**Supporting Information S1**).

3.3.1. Quality assurance

Most guidelines advise HMBs to implement DHM tracking and tracing systems and to conduct all operational processes based on Hazard Analysis and Critical Control Points (HACCP) and good manufacturing process (GMP) principles [1, 4, 5]. All HMBs implement at least one of the three systems; Approximately 40% of HMBs implement all three aforementioned systems, 30% implement two of the three systems, (HACCP & track and trace 7%, GMP & HACCP 2%, GMP & track and trace 21%) and another 30% only one of the three systems (HACCP 10%, GMP 9%, track and trace 11%).

3.3.2. Donor screening

The EMBA recommendations state that both verbal interviews and written health questionnaires should be performed as initial donor screening steps [1]. As a second step, all donors should undergo serological screening for a certain panel of diseases [1]. All HMBs indicated that donor selection was based on specific eligibility requirements, although with variation in requirements among the HMBs (**Table 1**).

Table 1. Parameters included in the donor screening processes of European HMBs (n=123).

	Screening parameters	**n (%)**
Lifestyle criteria	Smoking	121 (98)
	Alcohol	122 (99)
	Drugs of abuse	120 (98)
	Medicines	122 (99)
	HIV risk	116 (94)
	Extreme diets	71 (58)
Serological screening	Hepatitis B	123 (100)
	Hepatitis C	123 (100)
	HIV*	123 (100)
	HTLV‘	66 (54)
	CMV¤	58 (47)
	ALAT/ASAT ratio†	9 (7)
	After travelling (specific tests depending on country visited)	46 (37)
	Syphilis	35 (28)
	Chagas disease	6 (5)
	No need to undergo a screening process	3 (2)

*Human immunodeficiency virus, ‘Human T–lymphotropic virus, ¤Cytomegalovirus, †Aspartate aminotransferase / alanine aminotransferase.

Some requirements showed very little variation; Lifestyle criteria such as smoking, alcohol, drugs of abuse and medicines, serological screening for human immunodeficiency

virus (HIV), hepatitis B and C and the possibility of a donor being HIV infected within a specific period preceding the donation, are included in the donor screening processes of the majority of HMBs (>94%). Nonetheless, extensive inter– and intra–country variation was observed for cytomegalovirus (CMV) and human T–lymphotropic virus (HTLV) serological screening, screening for restricted diets (e.g. vegans), aspartate aminotransferase / alanine aminotransferase (ALAT/ASAT) ratio and testing after travelling to specific regions with increased risk of disease transmission.

Out of the 123 participating HMBs, seven HMBs dispense raw DHM only. For that reason, all donors are screened extensively. Serological screening for CMV is performed in six out of those seven HMBs, whereas five perform a serological HTLV screening. However, when adequate pasteurization is performed, CMV screening is not considered necessary [4, 5].

3.3.3. Start and duration of donation

In 75% of the HMBs, donors are allowed to donate milk from birth onwards while the remaining 25% allows donation only from a specific postnatal week onwards. The maximum duration of milk donation after delivery is specified in 63% of HMBs. A maximum duration of 6 months is set in 26% of HMBs, while 20% of HMBs allow donation for more than 6 months and up to one year (**Supporting Information S3**).

3.3.4. Expression and storage of human milk at home

Almost all HMBs (99%) provide donors with instructions on how to express, store and handle the milk. Most HMBs (76%) supply the donors with breast pumps for DHM expression (85% electrical, 15% manual).

EMBA's recently published recommendations state that HMBs should request their donors to freeze DHM as soon as possible, but at least within 24h (48h if collected and stored in a hospital refrigerator) [1]. Our data suggest that 75% of HMBs follow these recommendations. The maximum storage duration of DHM in a domestic freezer before transportation to HMBs varies from 1 week up to 6 months (**Table 2**).

3.3.5. Donor human milk handling at human milk banks

Upon arrival at the HMB, DHM should be checked for proper labelling (time of expression and donor identification should be clear) and whether it has remained frozen during transportation [1,4,5]. Our data show that about half of the HMBs (52%) have a home collection service, to ensure safe transportation. At the same time, 82% of HMBs check that DHM arriving at the HMB is both frozen and properly labelled. However, 18% of HMBs either accept DHM that arrives already partially thawed or they do not examine the milk's temperature at all.

In total, 62% of HMBs reported that unpasteurized DHM is kept in a refrigerator for up to 24h awaiting pasteurization or directly stored in a freezer, whereas 22% accept storage in the refrigerator up to 48h and 10% up to 72h. In total, 59% of HMBs set either 3 or 6 months as the maximum storage duration of unpasteurized DHM in the freezer (**Table 2**). Half of the HMBs (50%) reported that more than one thawing method for DHM is performed. Different methods could be combined due to practical reasons, such as time constraints or variations of the preferred equipment used (e.g. refrigerator, water bath, heating blocks, air bottle warmers).

Thawing DHM in a refrigerator is performed in 73% of HMBs, but only half of those HMBs use this method alone (**Figure 2**).Of the HMBs, 26% do not pool DHM, while 54% pool from a single donor and 20% pool from multiple donors (pools of 2–3 donors, n=11, pools of 4–8 donors, n=11 and no maximum number of donors specified, n=2).

Table 2. Maximum DHM storage duration at home and at the HMB, before and after pasteurization (n=123).

3.3.6. Pre– and Post–pasteurization donor human milk screening

There is a large variation in the microbiological screening practices of unpasteurized DHM among HMBs. EMBA recommendations suggest that all pools of milk should be tested before pasteurization, while every batch (referring to the bottles in a single pasteurization cycle) should be tested after pasteurization [1]. Our data suggest that before pasteurization, 23% of HMBs test microbiologically every single container of DHM while 33% test every sample of pooled DHM. Only 2% screen microbiologically both all single and pooled samples of DHM (**Supporting Information S4**).

A wide variation was observed in the microbiological criteria defining DHM acceptability before pasteurization (**Table 3**). In our study, 15% of the HMBs reported either not screening DHM microbiologically before pasteurization or that they are unaware of the criteria applied. DHM with more than 10^6 Colony–Forming Units (CFU) / ml for total viable bacteria counts (TVC) and 10^4 CFU/ml for *Staphylococcus aureus* is discarded in 13% of the HMBs, although this number refers to HMBs located in one country only. DHM with TVC>10^4 CFU/ml is discarded in 9% of HMBs, while in 8% of the HMBs, DHM is discarded when TVC>10^5 CFU/ml. The NICE guidelines specify that DHM should be discarded if TVC>10^5 CFU/ml or >10^4 CFU/ml for *Enterobacteriaceae* or *S. aureus*, which is followed by 8% of HMBs [5]. The EMBA recommendations suggest accepting DHM containing ≤10^5 CFU/ml non–pathogenic organisms and no pathogens for each DHM pool tested before pasteurization [1], which is done by 7% of HMBs. The applied criteria varied greatly, not only between but also within countries. HMBs from only two countries (out of the eight countries that were represented by n >3 HMBs in this study), follow a specific guideline with adherence ≥60% per country.

Microbiological testing after pasteurization is always performed in 56% of HMBs and regularly in 27%, where regularly includes once a month, every 10 pasteurization cycles, only when there are concerns about the processing, or when new equipment or employees are introduced. Microbiological testing after pasteurization is never performed in 11% of HMBs, while 6% do not pasteurize DHM.

After pasteurization, 62% of HMBs accept only DHM with no detected microbial growth. Pasteurized DHM with TVC≤10 CFU/ml is accepted in 13% of HMBs, while 8% accept DHM with counts ≥100 CFU/ml or have no defined thresholds. The remaining 17% either do not pasteurize DHM (6%) or do not perform microbiological testing after pasteurization (11%).

Table 2. Maximum DHM storage duration at home and at the HMB, before and after pasteurization (n=123).

		Responses	n (%)
Storage at home	**Maximum storage duration in a refrigerator (before freezing)**	0h–Immediate freezing	16 (13)
		1h–6h	8 (7)
		12h	9 (7)
		24h	57 (46)
		48h	26 (21)
		72h	3 (2)
		No handling at home	2 (2)
		Other	2 (2)
	Maximum storage duration in a freezer	1 week	9 (7)
		2 weeks	19 (15)
		1 month	14 (11)
		2 months	4 (3)
		3 months	24 (20)
		4 months	17 (14)
		6 months	17 (14)
		N/A	6 (5)
		Not specified	5 (4)
		Other	8 (7)
Storage at the HMB before pasteurization	**Maximum storage duration in a refrigerator**	0h–Immediate freezing	32 (26)
		12–14h	7 (6)
		24h	35 (28)
		48h	27 (22)
		72h	11 (9)
		Don't know	5 (4)
		Other	6 (5)
	Maximum storage duration in a freezer	1–2 weeks	5 (4)
		1–2 months	14 (11)
		3 months	43 (35)
		4 months	18 (15)
		5 months	2 (2)
		6 months	28 (23)
		> 6 months	2 (2)
		N/A	7 (6)
		Don't know	3 (2)
		Other	1 (1)
Storage at the HMB after pasteurization*	**4 to 5 °C**	24h	4 (3)
		48h	1 (1)
		72h	1 (1)
	–18 to –30 °C	3 months	39 (34)
		4 months	1 (1)
		6 months	58 (50)
		8–9 months	2 (2)
		2 years	1 (1)
		Don't know	1 (1)
	–80 °C	1 year	5 (4)
	Don't know–N/A		3 (3)
Total duration of DHM storage in a freezer (months)*	**–18 to –30 °C**	Minimum	2
		Maximum	49
		Mean ± SD	10.3 ± 5.61
	–80 °C	Minimum	13.38
		Maximum	18
		Mean ± SD	15.7 ± 1.97

*HMBs that do not pasteurize DHM are not included (n=7). SD, standard deviation.

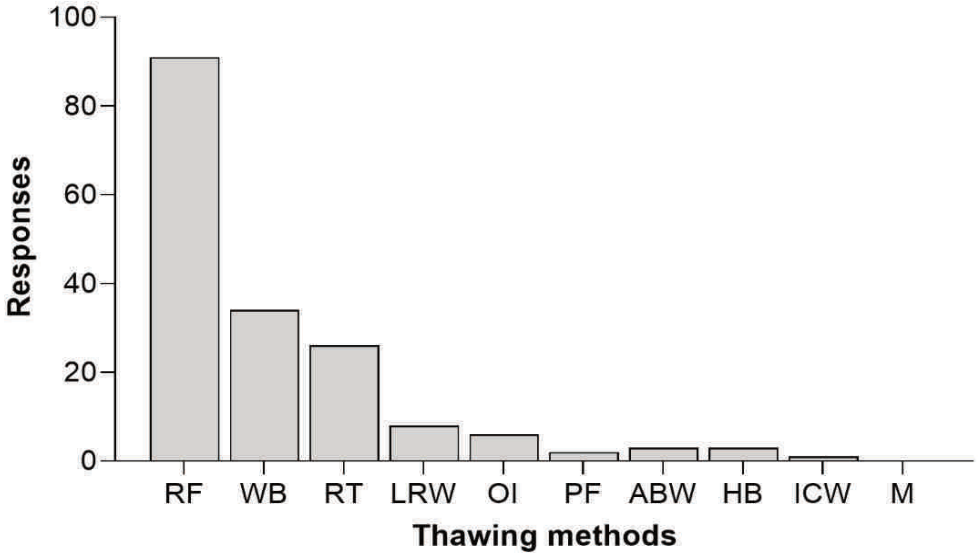

Figure 2. DHM thawing methods (RF=refrigerator, WB=water bath, RT=room temperature, LRW=lukewarm running water, OI=orbital incubator, PF=pasteurizer function, ABW=air bottle warmers, HB=heating blocks, ICW=Immersion in cold water, M=microwave). Answers are presented in absolute values. The participants could select multiple categories, in case multiple thawing methods were included in their practices.

Table 3. Microbiological criteria defining DHM acceptability before pasteurization (n=123).

Responses	**n (%)**
Total flora $\leq 10^2$ CFU/ml	2 (2)
Total flora $<10^3$ CFU/ml	3 (2)
Total flora $<10^4$ CFU/ml	11 (9)
Total flora $<10^5$ CFU/ml	10 (8)
Total flora $<10^5$ to $<10^4$ CFU/ml	3 (2)
Total flora $<10^6$ CFU/ml	6 (5)
Total flora 10^3–10^5 CFU/ml, other flora $<10^3$ CFU/ml	2 (2)
Total flora $<10^5$ CFU/ml, other flora $<10^3$ CFU/ml	2 (2)
Total flora $<10^5$ CFU/ml, pathogens=0 CFU/ml	2 (2)
Total flora $\leq 10^5$ CFU/ml, *S. aureus* $\leq 10^4$ CFU/ml	2 (2)
Total flora $\leq 10^5$ CFU/ml, *S. aureus* and *Enterobacteriaceae* $\leq 10^4$ CFU/ml (NICE guidelines)	10 (8)
Total flora $\leq 10^5$ CFU/ml, *S. aureus* and *Enterobacteriaceae* $\leq 10^4$ CFU/ml, *Bacilli*=0 CFU/ml	2 (2)
Total flora $<10^5$ CFU/ml, *S. aureus* $<10^4$ CFU/ml, *Coliforms* $<10^3$ CFU/ml	2 (2)
Total flora $\leq 10^6$ CFU/ml, *S. aureus* and *Enterobacteriaceae* $\leq 10^4$ CFU/ml, *Bacilli*=0 CFU/ml	2 (2)
Total flora $<10^6$ CFU/ml, *S. aureus* $<10^4$ CFU/ml [a]	16 (13)
Only when *S. aureus* $<10^4$ CFU/ml [b]	9 (7)
Only when pathogens $<10^4$ CFU/ml	2 (2)
DHM is assessed by the dornic acid test [c]	4 (3)
Swedish guidelines [d]	2 (2)
Not tested/Don't know	18 (15)
Other [e]	13 (11)

[a, b, c] This criterion is applied by HMBs located in one country only (n=3 countries, one criterion per country), [d] refers to the exact response received (acceptance criteria were not specified in detail), [e] HMBs with different individual acceptance criteria (n=13).

3.3.7. Donor human milk treatment

Holder pasteurization (62.5°C for 30 minutes) is recommended for DHM treatment. The ideal process should consist of a rapid heating phase, followed by a phase where the temperature remains constant, and finally a rapid cooling phase [1, 4, 5, 9]. Our findings show that DHM is heat treated in 94% of HMBs. Four HMBs in Norway, two HMBs in Germany and one HMB in Sweden represent the remaining 6% (n=7) that do not pasteurize DHM. DHM is heated at 62.5°C for 30 minutes in 95% of the HMBs that pasteurize DHM, while slightly different parameters (60–64°C for 30–65min, n=5 and 75°C for 15sec, n=1) are applied by the remaining 5%. The majority of HMBs (70%) reported using standard pasteurizers, with water as the heating medium. Shaking water baths and dry heating pasteurizers are lesser used (11% and 11%, respectively) and 8% did not specify pasteurizer design.

The same volume of DHM is included in every bottle within a pasteurization cycle by 66% of the HMBs. Of the remaining 34% of HMBs that pasteurize different DHM volumes within the same cycle, 6% answered that volumes depend on their needs, on available bottle sizes or that they are not aware of the volumes used. Differences in DHM volume ranging from 40ml to 90ml within the same pasteurization cycle were reported by 16% of HMBs and from 100ml to 210ml by 12% of HMBs.

The time required to raise the temperature of DHM to the pasteurization temperature (heating up time) and the cooling down time , which are important factors in processing efficacy, showed large differences among HMBs; Reported durations ranged from 10 to 120 minutes and from 5 to 110 minutes respectively, while the total processing time, which corresponds to the sum of the heating up time, the holding time and the cooling down time, ranged from 20 to 200 minutes (**Figure 3**). This could be attributed to the combination of different pasteurizer designs, DHM volumes and variations in the execution of the cooling phase. Lastly, 12% of HMBs do not monitor the temperature/time progression during the pasteurization process.

The cooling phase is automatically performed by the pasteurizer in 78% of HMBs and manually in the remaining 22% of HMBs, e.g. with iced water baths (n=10), freezers (n=3), refrigerators (n=4), blast chillers (n=7) or at room temperature (n=2). The majority of HMBs (68%) cool DHM to a temperature between 2 and 6°C.

3.3.8. Post–pasteurization storage

Pasteurized DHM is stored at –18°C to –30°C in 88% of HMBs. Almost all (96%) of those HMBs, store pasteurized DHM for 3 to 6 months while 3% exceeds this storing period.
Only 5% keep pasteurized DHM for 1–3 days at a refrigeration temperature (**Table 2**). The overall storage duration of DHM in a freezer (in a domestic freezer and in a HMB before and after pasteurization) was largely different among HMBs. The different storage durations applied are shown in **Table 2**.

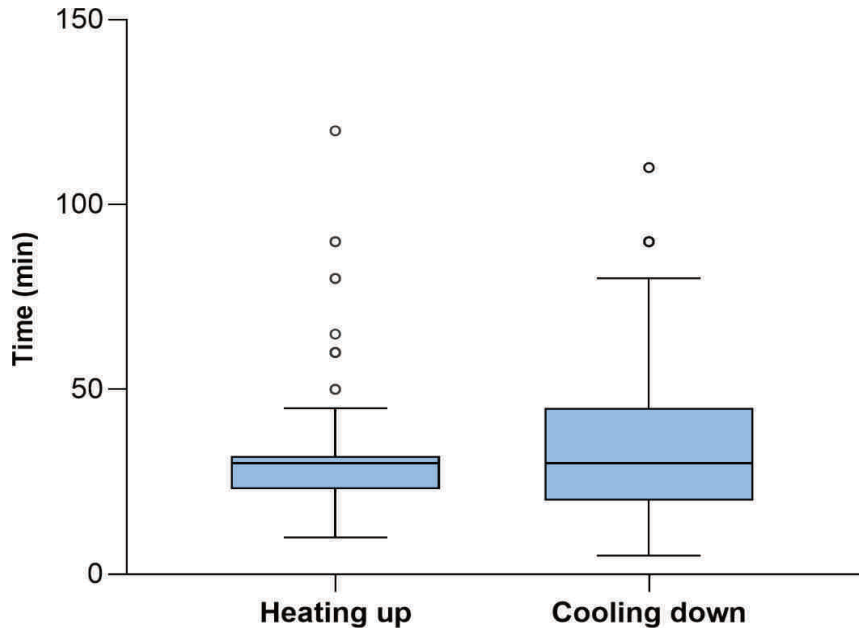

Figure 3. Heating up times to pasteurization temperature (n=103. "Don't know", n=13) and duration of the cooling cycle (n=106. "Don't know/Not controlled", n=10).

3.4. Discussion

Our findings showed a huge variation in the practices currently applied across European HMBs. Diversity of practices was observed not only between but also within countries, indicating that even when national guidelines existed, actual practices differed.

One of those practices was the maximum storage time in a freezer before pasteurization. This reflects the variation in the published recommendations which ranges from 1–12 months [4]. Similarly, on a global level, the regulations established by the National Sanitary Surveillance Agency (ANVISA) in Brazil, indicate 15 days as the recommended maximum DHM storage time at a temperature of –3 °C, while the recommendation from the Human Milk Banking Association of North America (HMBANA) is a maximum of 3 months, at –20°C [10, 11]. Prolonged storage duration (>3 months) could enable HMBs to secure adequate DHM supplies and reduce the disposal of expired DHM. However prolonged storage could also impact the quality of DHM. Studies investigating the effect of frozen storage (1–9 months) on specific proteins, report contradictory results (**Supporting Information S4**)[12–21]; Freezing DHM for 3 months at –20°C has been found to cause a minimal loss of its biological activity [12], but a significant decrease in lactoferrin levels has been also reported [13, 18]. On the contrary, one study found no effect on lactoferrin and SIgA levels after 9 months at –20°C [19]. Freezing pasteurized DHM at –20°C for 8 months did not decrease the macronutrient or energy content [20].

In conclusion, storage of DHM at –20°C for a maximum of 3 months seems to be safe without substantial loss of quality of the DHM. Probably a longer storage time can be applied, although more data are needed to make such a recommendation.

After storage of frozen DHM, thawing methods vary among HMBs. This is consistent with the existing recommendations, as not one specific thawing method is currently recommended. Thawing DHM in a refrigerator, in a water bath, at room temperature, under

running lukewarm water or with special thawing devices are all methods described in published guidelines (**Supporting Information S5**), thus including both slow and quick thawing methods [1, 5, 24–29]. The Brazilian regulations additionally allow thawing DHM in a microwave, but only when the exposure time for specific DHM volumes has been calculated based on the equipment specifications, size and shape of the bottles, so that DHM temperature does not exceed 5°C. According to the HMBANA guidelines, the DHM temperature while thawing should remain below 7.2°C, while EMBA recommendations specify that DHM temperature should not exceed 8 °C [10, 11]. A considerable risk when thawing DHM at room temperature or higher is bacterial growth [4]. When thawing DHM in a water bath or under running water, additional precautions should be taken to avoid submersion and cross–contamination through ingress of water in the event of the containers not being properly sealed [4]. Therefore, we propose that guidelines allowing such methods should extensively describe the monitoring procedure as well as all potential hazards.

Overall, since thawing can affect both the quality and the safety of DHM, certain practices should be preferred. Refrigeration overnight is considered as optimal, as no significant increase in bacterial counts for 24h has been reported [4, 30, 31]. Thawing DHM with waterless defrosting devices could be another option, as the risk of cross–contamination due to contact with water is eliminated while at the same time quicker thawing times are achieved [32]. As such devices can be conveniently used in HMBs, further research is needed in order to conclude on their effects on DHM quality.

Most guidelines recommend pooling of unpasteurized DHM from a single donor only [1, 5, 25, 29]. However, some guidelines also mention that multi–donor pools may be acceptable, but only from a limited number of donors (**Supporting Information S6**) [4, 26, 28]. Multi–donor pools are also allowed in other non–European published guidelines such as the Brazilian and the HMBANA guidelines ([10, 11]). In our study, 25 HMBs from various countries use multi–donor pools. One reason for using multi–donor pools could be the compensation for possible nutritional differences among donors, although nowadays, both nutrient analyses using human milk analyzers and individualized fortification can be performed. Pooling also enables smaller volumes of DHM to be used sooner, thus reducing pre–pasteurization storage times. To avoid microbial contamination and to ensure donor traceability, future guidelines should extensively describe the practices that should be followed if pooling is applied.

For DHM treatment, holder pasteurization is performed in almost all participating HMBs, with the exception of a few HMBs in Germany and Sweden, and the majority of HMBs in Norway. This method effectively inactivates DHM microbial contaminants, but the specific time–temperature combination used may negatively affect the activity of several DHM components [9]. Ensuring rapid heating up and cooling down is also of crucial importance; Since DHM bioactive components start to be significantly damaged from 58°C, the time DHM is heated above this temperature should be limited [9, 33]. In addition, optimized pasteurizers with shorter plateau duration and better temperature control during a cycle have been shown to better preserve SIgA, lactoferrin and lysozyme in DHM [34]. However, no recommendations are currently available regarding the maximum heating up time. Only the Brazilian regulations include detailed information on how to calculate the heating up time, based on the DHM volume, type and number of bottles used. The regulations additionally specify that all bottles

should contain the same volume of DHM and the starting temperature should be stable and around 5 °C. A table of the calculated heating up times for all different DHM volumes used in the HMB should then be created [11].

In addition, a rapid cooling down would minimize spore germination. To avoid bacterial proliferation, a temperature drop from 62.5°C to 25°C in 10 minutes is suggested [4]. Moreover, a total of 20 minutes to reach a final DHM temperature ≤ 8°C has been recommended [26]. Although temperatures <10°C are mostly suggested [1, 4, 5, 26, 35], no consensus currently exists over time and temperature.

Our data show that DHM is at present exposed to slow heating up and cooling down phases, which is in contrast with the recommended rapid pasteurization performance. The wide range of reported heating up and cooling down times could be due to the different pasteurizer designs, the final cooling temperature, and the differences in DHM volume within one pasteurization cycle. Dry heating pasteurizers seemed to expose DHM to longer total processing times, but as the majority of those pasteurizers do not include an automated cooling down phase, this is mostly dependent on how the cooling phase is performed (**Supporting Information S7**).

Due to the various practices applied, recommending a single practice would be challenging. However, additional recommendations on pasteurization efficacy can be added to the existing guidelines. A recommendation on the optimal duration of both phases could facilitate the standardization of pasteurization.

Bacteriological screening practices of DHM were quite variable both between and within countries in our study. This is in line with the EMBA's Guideline Working Group findings, where no consensus could be derived for either the defined criteria or for the frequency of testing [1]. More than half of the HMBs reported testing DHM only regularly (e.g. once a month). Interestingly, stricter practices were not applied even in HMBs performing multi–donor pools, thus increasing the risk of administrating DHM that does not meet the acceptance criteria. EMBA's recommendations (Test all DHM pools before pasteurization and accept DHM with $\leq 10^5$ CFU/ml of non–pathogens, test each batch after pasteurization and accept only DHM with no detected microbial growth) could be further adopted in order to increase the safety of the recipients. Regarding donor screening, the recruiting criteria should be flexible and adaptable to country–specific infectious diseases risk factors and the distribution of health–related events worldwide.

3.5. Conclusions

This study investigated actual human milk banking practices among European HMBs, with a high number of participants. Our findings highlight the wide variability covering most human milk banking practices in Europe, especially with regards to the DHM processing and bacteriological screening. When practices were evaluated based on both national and international guidelines, adherence was low, specifically with respect to the application of specific control systems, DHM storage, thawing, processing and screening. However, since variation in certain practices can exist without posing any safety risk, concluding on whether the observed variations have a negative impact on actual DHM quality and safety, remains a high priority. Risk assessment strategies may further assist in evaluating the effect of this variability, while future research may also focus on further analyzing the causes of these

variations. More extensive guidelines should therefore become available, while the need for developing guidelines covering all essential steps in DHM handling with large variations in execution such as DHM processing and storage, is of particular importance.

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Competing interest

The authors have declared that no competing interests exist.

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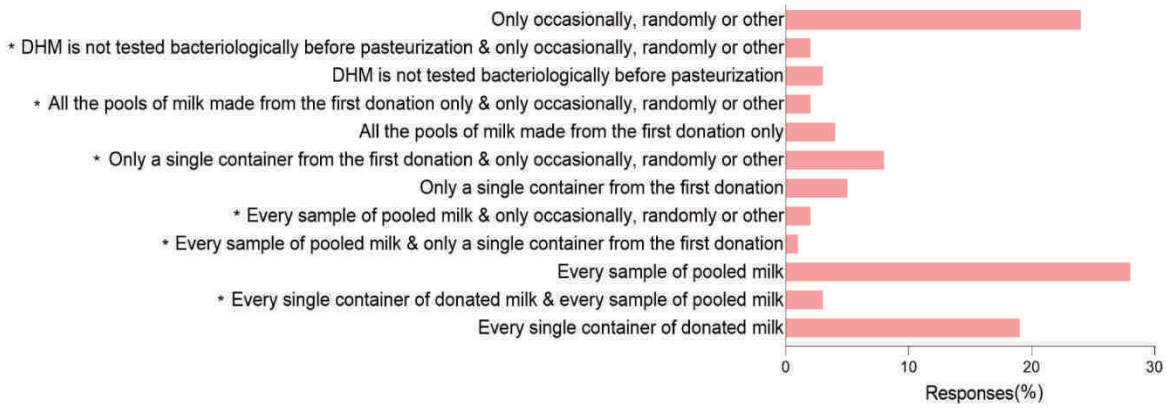
Supplementary Appendix

The study's minimal underlying data are available as a **Supporting Information file S7**. **Supporting information S2, S3**, **S6** and **S7** can be found online at:

https://doi.org/10.1371/journal.pone.0256435

Supporting Information S1. Human milk banks contacted and response rate.

Country	Number of contacted HMBs	Completed responses	Response rate per country	Responses per country/Total responses
France	34	22	65%	18%
Italy	38	15	39%	12%
Germany	22	14	64%	11%
Spain	15	14	93%	11%
Poland	16	13	81%	11%
Norway	12	10	83%	8%
Sweden	28	8	29%	7%
United Kingdom	16	7	44%	6%
Austria	5	2	40%	2%
Belgium	4	2	50%	2%
Greece	2	2	100%	2%
Russia	3	3	100%	2%
Switzerland	7	2	29%	2%
Bulgaria	1	1	100%	1%
Lithuania	2	1	50%	1%
Netherlands	1	1	100%	1%
Estonia	1	1	100%	1%
Finland	1	1	100%	1%
Portugal	1	1	100%	1%
Denmark	2	1	50%	1%
Romania	1	1	100%	1%
Serbia	1	1	100%	1%
Croatia	1	0	0%	0%
Czech Republic	1	0	0%	0%
Total	**215**	**123**		



Supporting Information S4. Frequency of DHM microbiological testing before pasteurization. Multiple selection of answer options was possible; For all HMBs that selected more than 1 option, combined categories were created. *Combined categories

Supporting Information S5. Effect of frozen storage (-20°C and -80°C) on DHM components, before and after pasteurization.

		No loss	**Decreased**	**Increased**
Before pasteurization	-20°C, 1 months	Lactoferrin[16]	Lysozyme, sIgA, Lactoperoxidase[16]	
	-20°C, 3 months	Lactoferrin, lysozyme, IgG, IgA, C3[17] Vitamin E, vitamin C, fatty acids[19] Bactericidal activity against *P.aeruginosa*[20] Lysozyme, Protease, lipase, B vitamins, lipids[9]	Lactoferrin (↓ 37%)[13], (↓ 55%)[10] Bactericidal activity against *E.coli*[20] Lactoperoxidase activity[9] Fat content[11]	Dornic Acidity [18]
	-20°C, 5 months	Vitamin E, vitamin C, fatty acids[19]		
	-20°C, 6 months	IgA, EGF, IL-8, TGF-β2, TGF-β1, TNF-RI, TNF-α, IL-6, IL-10[12]	Lactoferrin (↓ 46%)[13], (↓ 65%)[10] Bioactivity of lactoferrin[10]	
	-20°C, 8 months	Vitamin E, fatty acids[19]	Vitamin C[19]	
	-20°C, 9 months	Total protein, fat, lactoferrin, sIgA, osmolality[14]		Nonesterified fatty acids[19]
	-20°C, 12 months	Vitamin E, fatty acids[19]		
	-80°C, 3 months	Vitamin E[19] Bactericidal activity against *P. aeruginosa, E. coli*[20]		
	-80°C, 5 months	Vitamin E, vitamin C, fatty acids[19]		
	-80°C, 6 months	IgA, EGF, IL-8, TGF-β2, TGF-β1, TNF-RI, TNF-α, IL-6, IL-10[12]		
	-80°C, 8 months	Vitamin E, vitamin C, fatty acids[19]		
	-80°C, 12 months	Vitamin E, fatty acids[19]	Vitamin C[19] IgA, IL-8,TGF-β1[12]	
After pasteurization	-20°C, 3 months	Dornic Acidity [18]		
	-20°C, 8 months	Macronutrient and energy content [15]		

Chapter 4

High–Temperature Short–Time preserves human milk's bioactive proteins and their function better than pasteurization techniques with long processing times

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Abstract

Donor human milk is generally processed by holder pasteurization (HoP) at 62.5°C for 30 min. This temperature–time combination is sufficient for eliminating pathogens in donor milk, but also negatively affects several bioactive milk components. Long heating up times may further affect the bioactive properties of pasteurized milk. High–Temperature–Short–Time (HTST), a treatment with shorter processing times (72°C for 15 sec), was investigated as a suitable alternative to HoP. In addition, pasteurization methods that follow the same temperature regime but with varying heating up times were compared. Human milk samples form four different donors were combined into one pool, which was then used to perform all analyses. The effects of these methods on the levels and functionality of immunoglobulin A, lactoferrin, lysozyme and bile salt–stimulated lipase, were evaluated with LC–MS/MS–based proteomics and activity assays, while the pasteurization efficacy was evaluated with an alkaline phosphatase test. HoP, a treatment with long processing times, times, caused the highest reduction in all proteins studied (reduced by 50–98%). Compounds such as lactoferrin and bile salt–stimulated lipase that are more sensitive to heat treatments were better retained with HTST, but their levels and functionality were still significantly lower than those of untreated donor milk (52% and 81% reduction of lactoferrin and bile salt–stimulated lipase activity, respectively). Our findings showed that a treatment with considerably shorter processing times, such as HTST, may reduce the thermal damage caused to the bioactive proteins compared to HoP, without affecting pasteurization efficacy. Since the vast majority of the donor human milk banks that are currently operating on a global level apply HoP to donor milk, our findings may provide relevant information for the optimization of donor milk processing.

Keywords: Protein functionality; donor human milk; milk processing; holder pasteurization; HTST

4.1. Introduction

Tailored to each infant's need, mother's own milk represents the optimal source of neonatal nutrition. The unique composition of human milk (HM) promotes healthy infant development and growth, while the numerous bioactive factors it contains, protect infants against infections and various diseases [1, 2]. HM has been shown for example to be bacteriostatic against a number or bacteria, which is mainly attributed to the functionality of immunoglobulin A (IgA), lactoferrin (LTF) and lysozyme (LYZ). LTF, amongst others, limits the availability of the iron required for the growth of iron–dependent pathogens, LYZ disrupts cell walls in gram–positive bacteria, and secretory IgA is generally considered as the main antibody system in HM. Synergistic effects of these proteins have also been reported [3–5]. Especially for premature infants, when mother's own milk is not available, donor human milk (DHM) represents the best alternative form of nutrition. Current evidence suggests that DHM protects against necrotizing enterocolitis when compared to infant formula, while its provision is correlated with improved long–term outcomes in preterm infants [2, 6]. DHM should be provided though established human milk banks (HMBs) that can ensure its safety [7].

Before provision, HMBs subject DHM to holder pasteurization (HoP), a low–temperature long–time heat treatment at 62.5°C for 30 min [8]. HoP effectively inactivates

potential viral and bacterial agents, and it is known as the method recommended for DHM treatment in all international human milk banking guidelines [8, 9]. However, this method has been shown to cause substantial losses in various bioactive milk components, due to the thermally induced denaturation occurring in these components [8, 10]. In fact, as previously reviewed, losses from 20–90% in the levels and functionality of immunoglobulin A (IgA), lactoferrin (LTF), and lysozyme (LYZ) have been reported after this treatment [10]. In addition, HoP results in the total inactivation of bile salt stimulated lipase (BSSL), a heat–labile enzyme involved in fat absorption and infant metabolism [10]. The reported lower growth rates in preterm infants fed with DHM that underwent HoP, may be the result of this detrimental effect [11].

Different devices are currently being employed by HMBs to perform HoP. Standard pasteurizers are most commonly used, with water as the heating medium [8, 12]. The ideal HoP process should be comprised of a rapid heating up phase to 62.5°C, a phase of constant temperature (30 min) and finally a rapid cooling down phase to <10°C [8, 9]. A recent study investigating human milk banking practices in Europe, revealed that in contrast to the recommendations on pasteurization performance, DHM is currently exposed to long processing times [12]. This could result in higher losses of DHM components. More specifically, losses of 1.6, 1.7, and 2.4% were documented for IgA, LYZ and LTF respectively, for every minute spent at 62.5°C [13]. Therefore, since the duration of the heat treatment and the temperature at which DHM is exposed highly affects the preservation of its bioactive components, ensuring short heating up times seems essential when using thermal techniques.

Various studies report that high–temperature–short–time (HTST) treatment may be a suitable alternative to HoP, as it was found to provide similar microbial reduction (e.g. in *E.coli*, *S.aureus*, *S. epidermidis*, *E. faecalis*, *P. aeruginosa*, *L. monocytogenes*, *S. agalactiae* and *C. sakazakii* counts) and to better retain the DHM bioactive proteins [14–19]. HTST is usually performed by heating the milk at 72°C for a duration of 15 sec. This method is well–established in the dairy industry, and it usually involves the rapid heating of a thin layer of milk in a continuous flow system [20]. The shorter treatment time as well as the shorter exposure time at the processing temperature may be the reason of the promising results reported after this treatment [8]. However, substantial information on whether shorter heating up times during pasteurization can positively influence the retention of the DHM bioactive components, is at present lacking.

Our aim was to determine 1. the effect of shorter heating up times and 2. the effect of shorter pasteurisation duration at a higher temperature on the preservation of DHM quality, with emphasis on the IgA, LTF, LYZ and BSSL levels and bioactivity by comparing pasteurization methods that follow the same temperature regime.

4.2. Materials and methods

4.2.1. Milk samples

DHM samples were collected from the Dutch Human Milk Bank (located at Amsterdam UMC, Amsterdam, The Netherlands). Written informed consent was received from all donors before recruitment. Donor screening, milk expression and collection was conducted as per standardized protocols that adhere to internationally published guidelines [9]. The samples were

expressed with a breast pump and were subsequently collected in disposable bisphenol A–free bottles (Sterifeed, Medicare Colgate Ltd, Devon, England). The samples were immediately placed in a freezer at –20°C and were transported to the HMB, at a temperature of –20°C [21]. All samples were placed in a refrigerator (4°C) overnight, before analysis. Next, milk samples from four different donors were combined into a pool, to ensure sufficient DHM amounts for all analysis. This pool was then divided into two aliquots (600 mL); one that was centrifuged at 6500×g for 30 min at 4°C (with rotor 16.250, Avanti Centrifuge J–26 XP, Beckman Coulter, USA) to remove the fat, and one that remained unskimmed. Then, both the unskimmed and the skimmed samples underwent heat treatment (with the methods mentioned in section 2.2), except for the untreated control samples. After treatment, all samples were cooled in an stationary ice–water bath and stored at –20°C. **Figure1** illustrates the experimental approach used in this study.

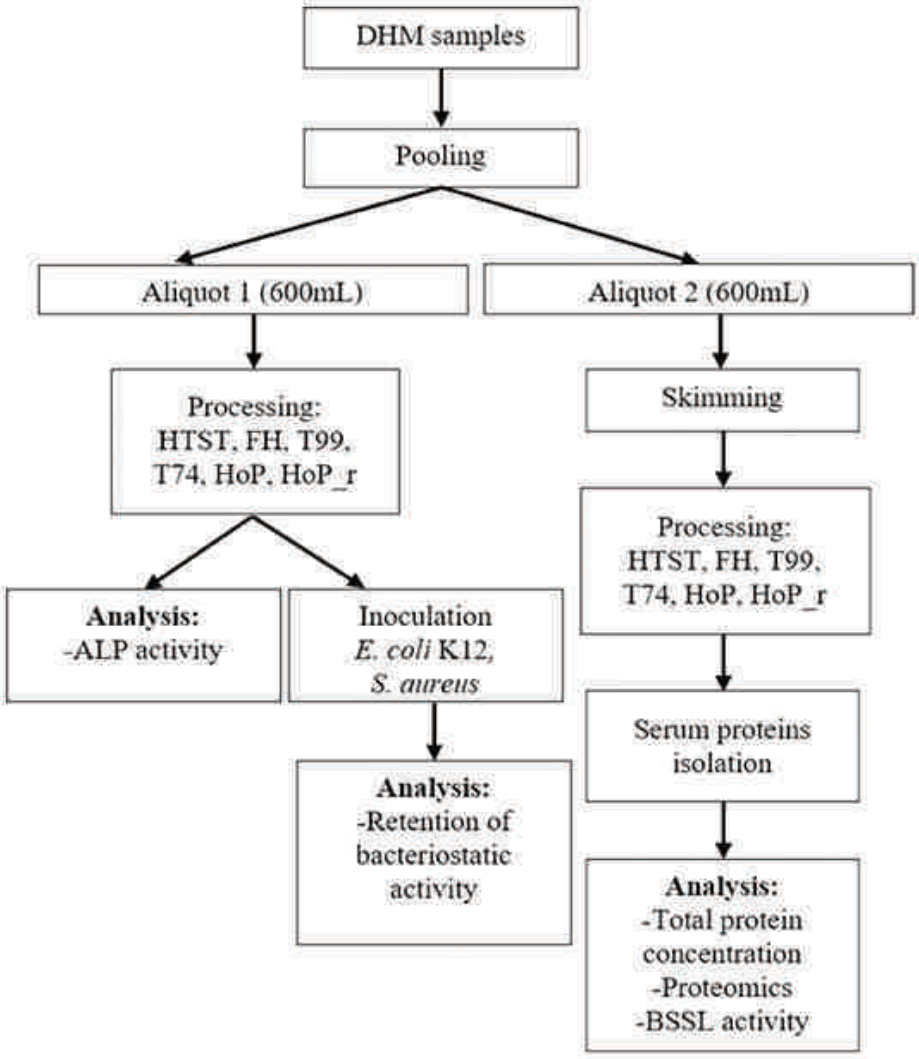

Figure 1. Schematic workflow indicating the experimental approach used. HTST, FH, T99, T74, HoP and HoP_r stand for high–temperature short–time, flash heating, high–temperature short–time with a thermomixer preheated at 99°C, high–temperature short–time with a thermomixer preheated at 74°C, holder pasteurization and holder pasteurization with rapid heating up–times, respectively.

4.2.2. Heat treatments

To compare the effects of processing times and temperatures on the DHM bioactive proteins, six pasteurization methods with different heating profiles were conducted; (1) HoP, (2) HoP with rapid heating up–times (HoP_r), (3) HTST, (4) flash heating (FH), (5) HTST with a thermomixer preheated at 99°C (T99), and (6) HTST with a thermomixer preheated at 74°C (T74). An overview of the different time–temperature profiles is provided as **supplementary material** (**Figure S1** and **Figure S2**).

4.2.2.1. Holder pasteurization

A 130 mL single–use polypropylene bottle (Beldico SA, Marche–en–Famenne, BE) was filled with 100 mL of DHM and was pasteurized at 62.5°C for 30 min, in a shaking water bath (SW22, Julabo GmbH, DE) at 150 rpm. To achieve a rapid cooling phase, the sample was placed in an ice–water bath after treatment, until reaching a core temperature of 4°C. The temperature was recorded by a temperature data logger RS PRO 1384 (RS Components B.V., The Netherlands). Three thermocouples were placed to monitor the temperature of the milk and of the two baths, during the whole process.

4.2.2.2. Holder pasteurization with rapid heating up–times

The HTST system described in section 2.2.2 was used to achieve rapid heating up to the pasteurization temperature (62.5ºC). The milk (50 mL) was pumped to the heating section and once its temperature reached 62.5ºC, it was transferred in a shaking water bath for 30 min, as described in 2.2.1.

4.2.2.3. High–temperature short–time

A laboratory scale pasteurizer was built to simulate continuous HTST pasteurization. The system included a peristaltic pump (Watson Marlow 505S, Hudson, MA, USA), as well as a heating, a holding, and a cooling section (**Figure 2**). All sections were connected to an RS PRO 1384 temperature data logger. The pump, which ran at a speed of 35 rpm, was connected with a plastic tube (ø 4mm) to a copper heating coil (810mm, ø 4mm), that was fully submerged into a water bath (heating section). A thermocouple was placed at the end of the coil to determine whether the milk (50mL) leaving the heating section indeed reached a temperature of 72°C. The coil was then connected to a plastic tube (ø 4mm) that remained submerged into a second water bath (holding section, 15 sec). A second thermocouple was placed at the end of the holding section. The milk passed then through a copper coil (1395mm, ø 4mm) which was submerged in an ice–water bath (2°C). Finally, a third thermocouple was linked with the coil to monitor the temperature at the end of the cooling phase. The cooled milk was then dispensed into a sterile bottle (Beldico SA, Marche–en–Famenne, BE).

4.2.2.4. Flash heating

The set up used for this treatment was based on previous reports [22, 23]. A 250mL Duran bottle (GL 45, DWK Life Sciences GmbH, DE) was filled with 100 mL of DHM and was placed into an aluminium pan (2 L) containing 450 mL of water. The water and the submerged DHM bottle were heated simultaneously using an electric hot plate, until the water reached 100°C.

The bottle was removed once the milk's temperature reached 71.5°C and was transferred into an ice–water bath (2°C). The temperatures of both milk and water were recorded by a temperature data logger (RS PRO 1384).

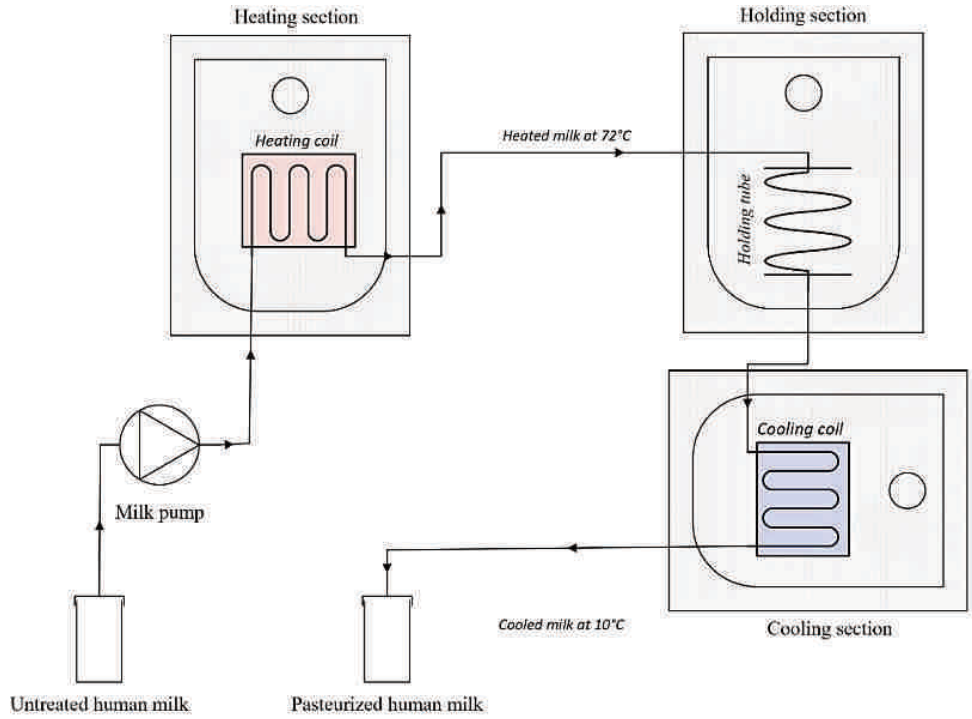

Figure 2. Schematic representation of the HTST system set–up.

4.2.2.5. Thermomixer preheated at 99°C and 74°C

A shaking thermomixer (12ml block, Eppendorf Thermomixer R Mixer, Hamburg, DE) was preheated to either 99°C or 74°C. Eight 12 mL Greiner tubes (Greiner Bio–One International GmbH, DE) , each filled with 10 mL of DHM, were placed in the thermomixer once the desired temperature was reached (99°C or 74°C). The samples were shaken during the whole treatment (800 rpm), while the temperature was monitored with a temperature data logger. When the core temperature of the milk reached 72ºC, the samples were removed from the thermomixer and after 15 sec, they were then immediately transferred in an ice–water bath (2ºC) for a rapid cooling down.

4.2.3. Native milk serum and total protein content

To obtain the native serum protein fraction from the skimmed DHM samples, caseins and denatured proteins were precipitated by acidifying the samples with HCl (1 mol/L) under stirring, until a pH of 4.6 was reached [24]. After leaving it for 30 min at 4°C to equilibrate, the samples were ultracentrifuged for 90 min at 100,000×g and at 30°C (Optima L–80, Beckman Coulter, USA). Next, the supernatant with the native serum protein fraction was collected and the pellet containing micellar casein and denatured proteins was discarded. Finally, total protein content was determined with the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, USA), as specified in the manufacturer's instructions.

4.2.4. Proteomics by liquid chromatography with tandem mass spectrometry (LC–MS/MS)

4.2.4.1 Filter aided sample preparation (FASP) for proteomics

Before LC–MS/MS measurements, the samples were prepared with the FASP method, as previously described [25]. Briefly, after the samples (1.0 µg/µL) were reduced with 15 mM dithiothreitol, they were first diluted by urea (8 M) in 100 mM Tris/HCl (0.1 M, pH 8.0) and then alkylated with the addition of 20 mM of acrylamide (0.2 M). Next, 100 µL of the alkylated samples were transferred to a Pall 3 K omega filter (10–20 kDa cut off, OD003C34, Pall corporation, Port Washington NY, USA) and were centrifuged for 30 min at 16900×g. The samples were then washed with 50 mmol/L NH_4HCO_3 (ABC) and were centrifuged again at the same conditions. The filter units were placed into new low–binding tubes (2 mL) and the samples were subjected to overnight digestion by the addition of 100 µL trypsin in NH_4HCO_3 solution (5 ng/µL). Next, a centrifugion step at 16,900×g for 30 min followed, and another one at the same conditions after the addition of 100 µL 1 mL/L HCOOH in water on top of the filter unit. Finally, 3 µL of 10% trifluoroacetic acid was added to the filtrate to adjust sample pH to around 3. Before injection into the LC–MS/MS system, all samples were stored at –20°C.

4.2.4.2. LC–MS/MS proteomics

All analyses were carried out by the department of Biochemistry at Wageningen University and Research. The parameters used were the same as previously reported [25]. The samples were directly injected on a 0.10*250 mm ReproSil–Pur 120 C18–AQ 1.9 µm beads analytical column prepared in house, at 800 bar. Elution of the peptides was done at a flow of 0.5 µL/min, using an acetonitrile gradient (9–34% acetonitrile in water with 1 ml/L formic acid in 50 min). The eluent was then ejected trough the tip of a needle with an electrospray potential of 3.5 kV. Full scan FTMS in positive mode between m/z 380 and 1400 were measured using Q Exactive HF–X mass spectrometer (Thermo Electron, San Jose, CA, USA). MS/MS scans of the twenty most abundant multiply charged peaks, were measured in data–dependent mode. MaxQuant software (v1.6.3.4) was used to analyze the obtained MS data, against the Uniprot human protein database and a database containing the sequences of common contaminants [26]. Protein modifications were set as propionamide (C) (fixed) and oxidation (M) (variable), while enzyme specificity was set for trypsin and a maximum of two missed cleavages, 20 ppm peptide tolerance first search, 4.5 ppm main search and 20 ppm MS/MS fragment match tolerance. Requirement for further analysis was the protein identification by a minimum of two peptides that had at least one unique and one unmodified peptide. Proteins detected in less than half of the samples as well as keratins and trypsin were removed from the final list of identified proteins.

4.2.5. BSSL activity

The assay used to determine BSSL activity was based on a previously published method, with minor modification [27]. DHM lipase activity is determined fluorometrically through the utilization of the two synthetic substrates; 4–methylumbelliferyl butyrate (4–MUB) and 4–methylumbelliferyl laurate (4–MUL). Defatted DHM samples were preincubated at 40°C for 3 min, under 800 rpm, in a ThermoMixer (SmartBlock 1.5 ml, Eppendorf, Hamburg, DE). A stop

solution of GuHCl (8 M) and HCl (1 M) in water was then used to stop the conversion of the added substrate and a neutralizing solution with Bis–tris (1M), NaOH (0.85 M) and EDTA (0.25 M) in water was added next to clarify the samples. The fluorescence released was measured by using a fluorimeter (SpectraMax ID3, Molecular Devices, San Jose, CA, USA) at an excitation of 355 nm and an emission of 460 nm.

4.2.6. Alkaline Phosphatase (ALP)

The method used for the detection of ALP was according to an international standard protocol (ISO/TS 6090|IDF/RM 82A:2004). Finally, ALP activity was measured in a p–nitrophenol calorimeter (Lovibond APTW/7, Tintometer GmbH, Dortmund, DE).

4.2.7. Bacteriostatic properties

In order to assess the effect of the different methods on the functionality of the three major HM antimicrobial proteins (IgA, LTF, LYZ), we evaluated the growth rate of two bacterial strains known to be inhibited by these proteins. Fresh cultures of *Escherichia coli K12* (DSM 498, DSMZ, Braunschweig, Germany) and *Staphylococcus aureus* (ATCC 6538, American Type Culture Collection, Manassas, USA) were prepared from frozen stocks in nutrient broth overnight at 37°C (CM0001, Thermo Fisher Scientific, Massachusetts, USA). Bacterial pellets were obtained after a centrifugation step of 10 min at 4000×g (Microcentrifuge 5890R, Eppendorf, Hamburg, Germany) and were subsequently dissolved in peptone physiological salt solutions (PFZ; Tritium Microbiology, The Netherlands). Optical density was determined by using a spectrophotometer (Cary 50 UV–Visible Spectrophotometer, Agilent Technologies, USA). Next, *E. coli* and *S. aureus* cultures were inoculated into untreated samples and samples that were first subjected to heat treatment with the different methods, to a concentration of approximately 10^3 colony forming units (CFU)/mL. DHM samples inoculated with *E. coli* were incubated at 37°C for 2 h and the samples inoculated with *S. aureus* for 4 h, at the same temperature. All samples were plated in duplicate onto selective media; violet red bile glucose agar for *E. coli* (CM0107B, Thermo Fisher Scientific, Massachusetts, USA) and mannitol salt agar for *S. aureus* (CM0085B, Thermo Fisher Scientific, Massachusetts, USA) and were then incubated overnight at 37°C. Bacterial counts were determined by colony counting (CFU/mL) while the growth rare per hour was measured as $ln(\frac{N_t}{N_0})/\ t$, were N_t = bacterial counts after either 2 h or 4 h of incubation, N_0 = bacterial counts immediately after incubation and t= incubation time.

4.2.8. Data analysis

GraphPad Prism software 8.0 (GraphPad Inc., La Jolla, CA) was used for data analysis and visualization. The effects of the different treatments were compared by ANOVA and Tukey's HSD for post–hoc tests. Protein retentions (%) were determined after dividing the concentrations after treatment by the concentration of untreated samples, multiplied by 100. The intensity based absolute quantification (iBAQ) values obtained with MaxQuant, were analyzed in Perseus software (v.1.6.2.1, Martinsreid, Germany). The iBAQ values are considered as suitable indicators for absolute protein concentrations, as the values refer to the sum of all peptide intensities divided by the number of theoretically generated tryptic peptides

[28]. Perseus was used to estimate significant differences in the protein pattern after treatment, by Student's t–tests with permutation–based false discovery rate (FDR) correction. The correlation between the levels of IgA, LTF and LYZ retained and the bacterial growth rate was also determined, by creating a correlation matrix with R version 3.4.0 [29]. A p–value < 0.05 was used to indicate significant differences among the compared groups. The analyses were performed in duplicate for each sample and all data are shown as mean ± standard deviation of two independent experiments.

4.3. Results

4.3.1. Temperature profiles

The temperature profiles of the different treatments were broken down into three sections: the heating up time to reach the pasteurisation temperature (referred to as heating up time), the time that DHM was held at this temperature, and the time required for DHM to cool down to 4°C (**Table 1**). The time DHM spent above 55°C was used as an indicator of the thermally induced protein denaturation that usually occurs above this temperature [30]. During HoP, the samples were exposed to temperatures above 55°C for about 44 min, which was the longest exposure observed among the different treatments. In contrast, HTST–treated DHM was exposed above this temperature for only 33 sec.

Table 1. Time–temperature profiles of the six processing methods. HTST, FH, T99, T74, HoP and HoP_r stand for high–temperature short–time, flash heating, high–temperature short–time with a thermomixer preheated at 99°C, high–temperature short–time with a thermomixer preheated at 74°C, holder pasteurization and holder pasteurization with rapid heating up–times, respectively.

Processing methods	Volume processed (mL)	Time above 55 °C (min)	Heating up time (min)	Holding time (min)	Cooling down time (min)
HTST	50	0.33	0.12	0.25	0.18
FH	100	5	9	0.25	7.5
T99	80	7.3	11	0.25	3.3
T74	80	32	40	0.25	2.3
HoP_r	50	31.25	0.10	30	16
HoP	100	44	27	30	16

4.3.2. Native milk serum protein concentration and quantitative analysis of the milk serum proteome

The total native milk serum protein concentration of the untreated and the treated samples is shown in **Figure 3**. Of the treatments tested in this study, only HoP caused a significant decrease in native protein concentration ($p<0.05$), when compared to the untreated samples. In addition to the total protein content, the native protein profile was assessed as well by LC–MS/MS. The impact of the different heat treatments on the DHM native protein profile was then visualized by a clustered heat map of the obtained iBAQ values **(Figure 4**). According to the clustering pattern (**Figure 4**), the native protein profile of the samples that were the longest exposed to temperatures > 55°C (samples treated with T74, HoP or HoP_r) differed the most from the

protein profile of the untreated samples.

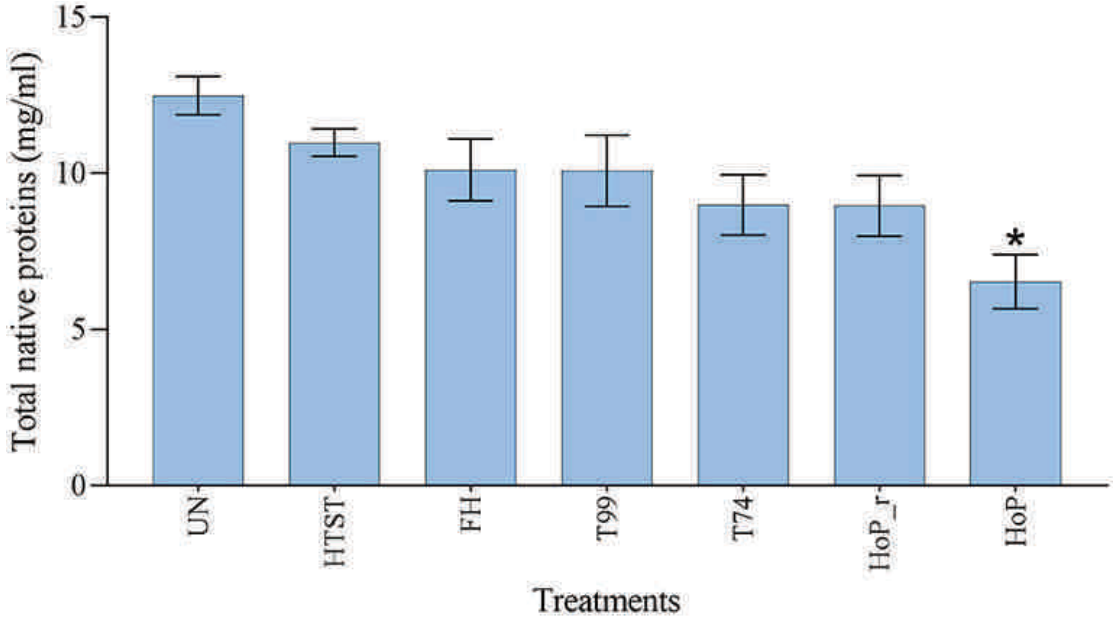

Figure 3. Total protein concentration (mg/ml) in native milk serum of untreated and the different heat treated DHM samples. The analyses were performed in duplicate for each sample and all values are presented as mean ± standard deviation of two independent experiments. *Expresses statistically significant difference to untreated samples ($p<0.05$). UN, HTST, FH, T99, T74, HoP and HoP_r stand for untreated DHM, and DHM treated with high–temperature short–time, flash heating, high–temperature short–time with a thermomixer preheated at 99°C, high–temperature short–time with a thermomixer preheated at 74°C, holder pasteurization and holder pasteurization with rapid heating up–times, respectively.

4.3.3. IgA, LTF and LYZ levels after processing

Overall, the retentions of the three proteins showed a decreasing tendency with increasing exposure time above 55°C. Compared to untreated DHM, FH, T99, HoP_r, T74 and HoP significantly reduced the IgA, LTF and LYZ levels ($p<0.05$), with average retention rates between 19% to 64% (**Figure 5**). HoP preserved IgA, LTF and LYZ levels the least, but HoP with rapid heating up times was shown to improve their retention, although the differences observed between the two methods were non–significant (mean ± SD retention rates of IgA, LTF and LYZ after HoP_r and HoP; 50±5% versus 44±4%, 26±18% versus 19±4%, 60±18% versus 50±6%, respectively, $p>0.05$). HTST, the treatment with the shortest processing times, was found to better retain the levels of the three proteins; the concentrations of IgA and LYZ were not significantly different from those of untreated samples (74±9% and 82±19%, respectively) but the concentration of LTF was significantly reduced (48±13% of LTF was retained, $p<0.05$). However, the concentration of LTF after HTST was significantly higher than after HoP and T74 ($p<0.05$). Overall, the data showed that the IgA levels were significantly higher after HTST than after HoP ($p<0.05$), but for LYZ, no significant differences were observed between the two treatments.

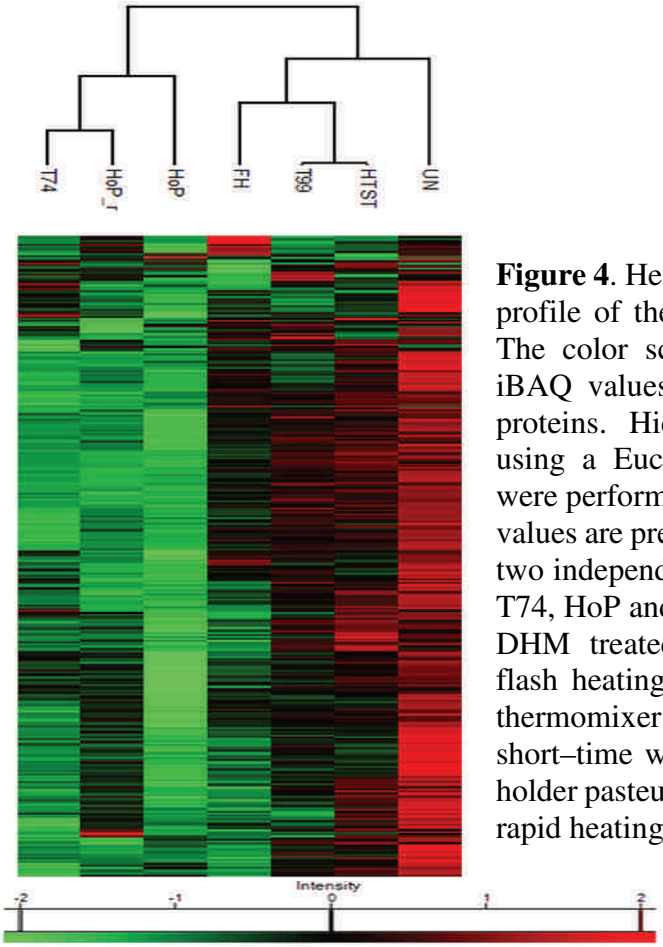

Figure 4. Heat map indicating differences in protein profile of the differentially heated DHM samples. The color scale is based on z–score normalized iBAQ values and each row represents individual proteins. Hierarchical clustering was performed using a Euclidean distance metric. The analyses were performed in duplicate for each sample and all values are presented as mean ± standard deviation of two independent experiments UN, HTST, FH, T99, T74, HoP and HoP_r stand for untreated DHM, and DHM treated with high–temperature short–time, flash heating, high–temperature short–time with a thermomixer preheated at 99°C, high–temperature short–time with a thermomixer preheated at 74°C, holder pasteurization and holder pasteurization with rapid heating up–times, respectively.

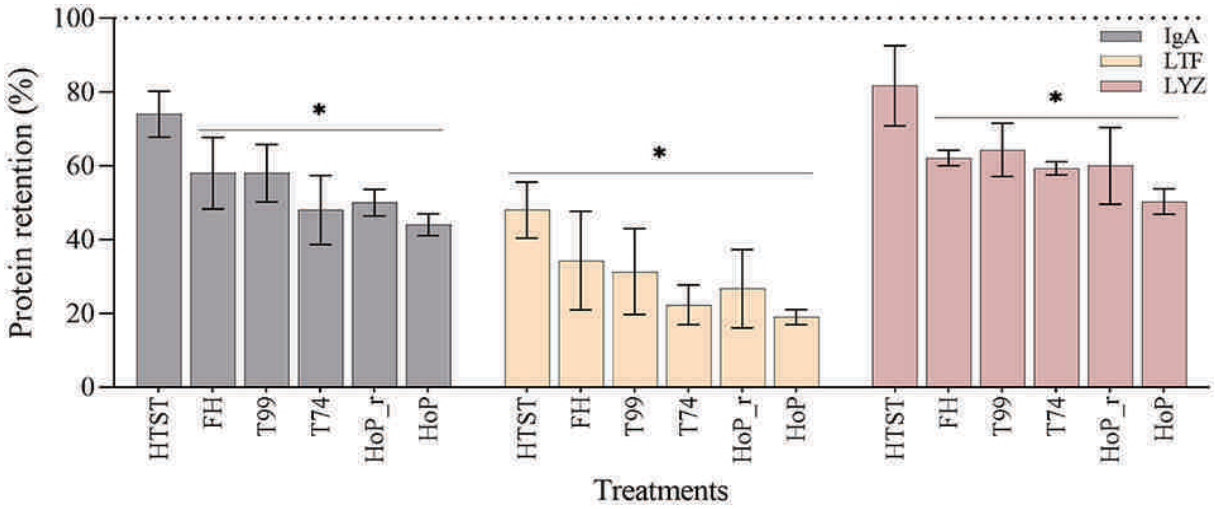

Figure 5. Effect of HTST, FH, T99, HoP_r, T74 and HoP on the IgA, LTF and LYZ content. Protein retention (%) was calculated based on the iBAQ intensities obtained by LC–MS/MS. The dotted line represents the untreated values (100%). The analyses were performed in duplicate for each sample and all values are presented as mean ± standard deviation of two independent experiments. * Expresses statistically significant differences to untreated samples ($p<0.05$). UN, HTST, FH, T99, T74, HoP and HoP_r stand for untreated DHM, and DHM treated with high–temperature short–time, flash heating, high–temperature short–time with a thermomixer preheated at 99°C, high–temperature short–time with a thermomixer preheated at 74°C, holder pasteurization and holder pasteurization with rapid heating up–times, respectively.

4.3.4. BSSL level and activity after processing

The effects of the different heat treatments on BSSL level and activity were determined by means of LC–MS/MS and an activity assay. All treatments caused a major reduction on the enzyme's level and activity, with respect to untreated DHM ($p<0.05$, **Figure 6**). BSSL was affected the most by HoP (LC–MS/MS, 2% and activity assay, 4%), but the values obtained after FH, T99, HoP_r and T74 were comparable to those of HoP ($p>0.05$). HTST retained significantly higher BSSL level and activity than the other treatments (LC–MS/MS, 9% and activity assay, 19%, $p<0.05$).

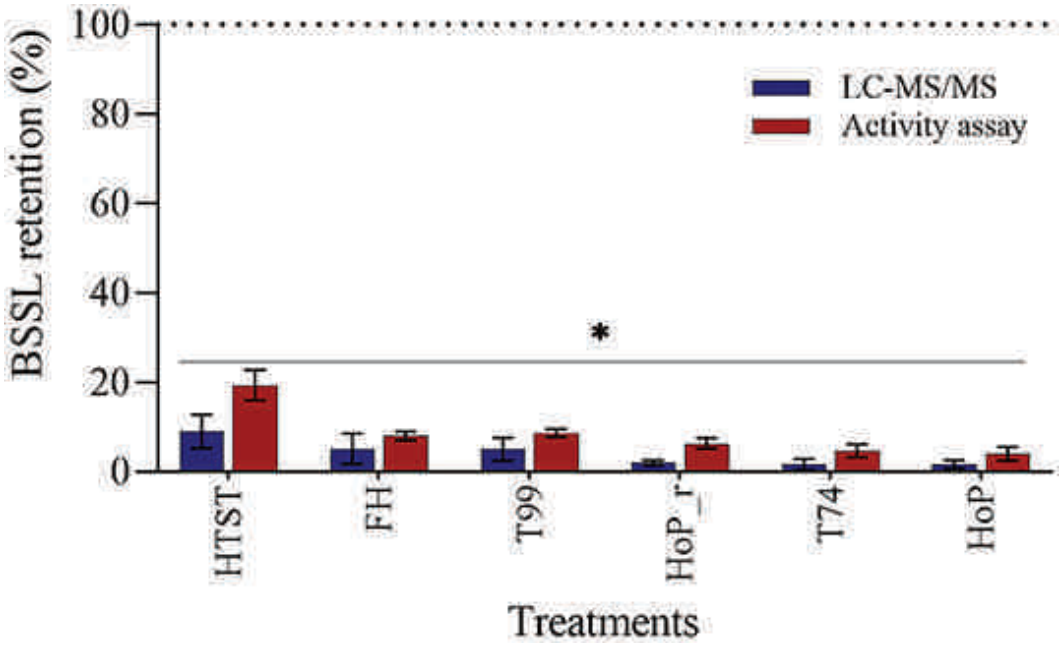

Figure 6. Effect of HTST, FH, T99, HoP_r, T74 and HoP on BSSL levels. BSSL retention (%) was calculated based on the iBAQ intensities and a lipase activity assay. Untreated values were set at 100% (dotted line). The analyses were performed in duplicate for each sample and all values are presented as mean ± standard deviation of two independent experiments. * Expresses statistically significant differences to untreated samples ($p<0.05$). UN, HTST, FH, T99, T74, HoP and HoP_r stand for untreated DHM, and DHM treated with high–temperature short–time, flash heating, high–temperature short–time with a thermomixer preheated at 99°C, high–temperature short–time with a thermomixer preheated at 74°C, holder pasteurization and holder pasteurization with rapid heating up–times, respectively.

4.3.5. ALP activity after processing

ALP is a very heat–sensitive enzyme and it is expected to be completely inactivated when the pasteurization is adequate [19]. Untreated DHM samples exhibited a mean ALP activity of 0.257 ± 0.049 U/ml, whereas all heat–treated samples were below the detection limit.

4.3.6. Retention of bacteriostatic properties after processing

To assess the impact of the different heat treatments on the DHM bacteriostatic capacity, the growth rates of *S. aureus* and *E. coli* were evaluated, in untreated and in heat–treated samples (**Figure 7**). The lowest growth rate for both strains was documented in untreated DHM (1.7 ± 0.36 and 3.6 ± 0.04–fold per hour, for *S. aureus* and *E. coli*, respectively), which indicates that

untreated DHM samples exhibited the highest bacteriostatic capacity among all samples ($p<0.05$). In contrast, HoP caused the highest reduction in bacteriostatic capacity (*S. aureus* and *E. coli* growth rate, 2.9 ± 0.01 and 5.53 ± 0.09–fold per hour, respectively, $p<0.05$). Compared to the untreated samples, HTST resulted in a comparable *S. aureus* growth rate (2.0 ± 0.17–fold per hour, $p>0.05$) but the *E. coli* growth rate increased significantly after this treatment (4.2 ± 0.07–fold per hour, $p<0.05$). Similarly, the bacterial growth rate was significantly increased after FH, T99, HoP_r and T74 ($p<0.05$). When compared to HoP, *S. aureus* growth was significantly lower after HTST, FH, T99, HoP_r and T74, but when *E. coli* growth was assessed, that was the case only for the samples after HTST, FH and T99 ($p<0.05$).

Considering that the IgA, LTF and LYZ levels decreased while the *S. aureus* and *E. coli* growth rate increased, a negative correlation between bacterial growth rate and the retention of the three major antimicrobial proteins is expected, as was indeed found (**Figure 8**). The strongest negative correlation was observed between bacterial growth rate and the levels of LTF and LYZ (LTF; r =–0.91 and r=–0.96, LYZ; r =–0.81 and r =–0.80, for *S.aureus* and *E.coli* respectively, $p<0.05$). The correlation between IgA levels and bacterial growth rate was weaker but still significant (*S.aureus*, r =–0.62 and *E.coli*, r =–0.51, $p<0.05$).

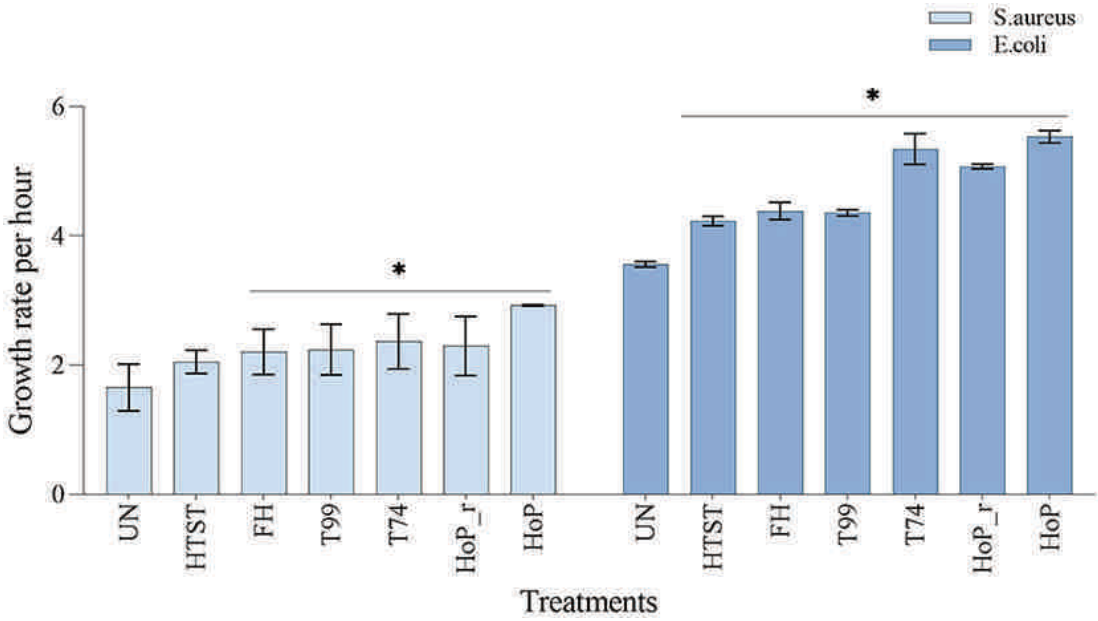

Figure 7. Growth rate per hour of *S. aureus* and *E. coli* in untreated DHM samples and after HTST, FH, T99, HoP_r, T74 and HoP. The analyses were performed in duplicate for each sample and all values are presented as mean ± standard deviation of two independent experiments. *Expresses statistically significant differences to untreated samples ($p<0.05$). UN, HTST, FH, T99, T74, HoP and HoP_r stand for untreated DHM, and DHM treated with high–temperature short–time, flash heating, high–temperature short–time with a thermomixer preheated at 99°C, high–temperature short–time with a thermomixer preheated at 74°C, holder pasteurization and holder pasteurization with rapid heating up–times, respectively

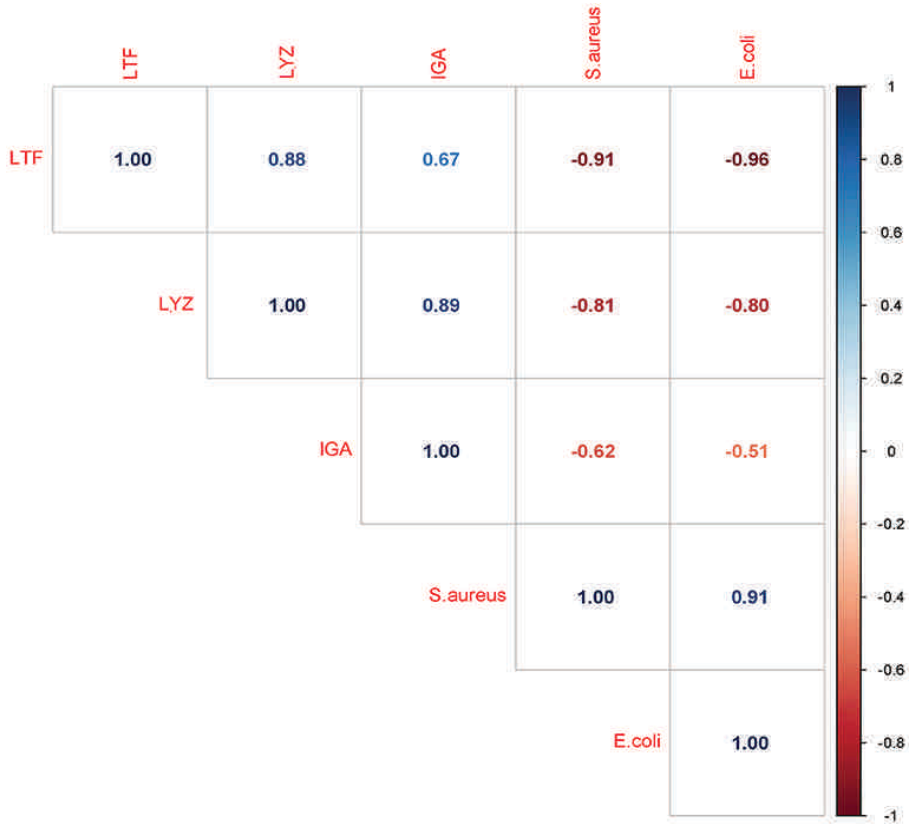

Figure 8. Correlation matrix of bacterial growth rates and IgA, LTF and LYZ iBAQ intensities. Each box includes a Pearson correlation coefficient (r value).

4.4. Discussion

The current study illustrates that both shorter heating up times and shorter duration of pasteurisation at a higher temperature preserved the levels and bioactivity of key DHM bioactive components better than HoP. In addition, the ALP assay showed that all tested time–temperature combinations resulted in the complete inactivation of the enzyme, indicating that sufficient heat load was applied.

4.4.1. Effects of processing times and temperatures on IgA, LTF and LYZ levels and activity

Our results confirmed the major impact of HoP on the DHM protein profile [16]. HTST, FH and T99, the treatments with the shortest processing times, seemed to better preserve the DHM protein profile when compared to HoP_r, T74 and HoP. Among the methods tested, HTST showed the least reduction in the levels of the studied bioactive proteins, while HoP showed the highest reduction in their levels. More specifically, IgA and LYZ levels after HTST were not significantly different to those of untreated samples, while LTF was significantly reduced after all thermal treatments. These differences were to be expected as DHM was exposed above 55°C the shortest after HTST (0.33 min) and the longest after HoP (44 min).

Our findings of the advantages of HTST over HoP in the retention of key DHM bioactive components are in line with the literature [14, 16, 19, 31–33]. However, considerable variations in the losses of IgA, LTF and LYZ after HTST are reported; 0–60% of IgA and 0–85% of LTF, while for LYZ, losses up to 40% and increases up to 28% were reported [32, 34–37]. Several reasons may explain these variations, including the fact that the extent of protein

denaturation depends both on their physicochemical characteristics and the nature of the thermal treatment [38]. The different HTST devices (e.g. laboratory or industrial heat exchangers, other benchtop devices or by immersion in thermostatically controlled water baths in bulk processes), the differences in holding times (5–25 sec) and temperatures (62–87°C) as well as differences in the methods of analysis in these studies (e.g. ELISAs, radial immunodiffusion assays, enzymatic activity assays, mass spectrometric methods) may have also contributed to different protein retentions documented [15–19, 32, 33, 35, 36, 39].

In respect to the impact of the different heat treatment parameters on DHM bioactive components, T74 caused the highest protein loss of the treatments following the same temperature regime as HTST (15 sec at 72°C). This could be attributed to the longer heating up time documented during T74 (40 min), which was the result of the small heat exchanging surface area and the small temperature differences between the heating medium and the desired pasteurization temperature. Similarly, HoP_r performed slightly better, although not significantly, than HoP. The possible explanation could be that HoP and HoP_r both follow the same holding regime (30 min at 62.5°C), but the heating up time is much shorter for HoP_r (0.10 min) than during HoP (27 min). When evaluating the performance of the two treatments with the shortest heating up times, HTST and HoP_r, it was clear that the considerably longer holding time during HoP_r was the reason of the higher protein damage caused. These observations suggest that the combination of processing parameters that leads to a prolonged exposure above temperatures 55°C is of crucial importance for the retention of the three studied proteins.

In accordance with our results, Buffin et al., (2018) also showed that an optimized HoP, with a mean plateau temperature of 1.5°C lower and duration of 11 min shorter than HoP, preserved higher amounts of IgA, LTF and LYZ [40]. In addition, Escuder–Vieco et al., (2018) found a 30% decrease in IgA concentration, regardless of the temperature–time combination used for HTST (5–25 sec at 70–75°C), while Mayayo et al., (2014, 2016) showed that the largest reductions in the IgA and LTF levels during HoP were documented during the first 5 min of treatment (45% and 70% for IgA and LTF, respectively), with the remaining 25 min of treatment caused <10% reductions [35, 42, 43]. For LYZ, studies showed contradictory results in the effect of thermal treatments due to its stable structure, which may be explained by the different analytical approaches used to measure its activity [17, 35, 36, 44, 45].

When the effect of the different heat treatment parameters on the DHM bacteriostatic capacity was assessed, a decrease of the bacteriostatic capacity with increasing exposure times above 55 °C was observed. Furthermore, the correlations observed between IgA, LTF and LYZ levels and the growth rate of *S. aureus* and *E. coli*, which are sensitive to these proteins, indicate that these proteins may have a significant role in retarding their growth. Of the treatments performed in the current study, HoP–treated DHM was found to exert the lowest bacteriostatic capacity, while the bacteriostatic capacity of HTST–treated DHM was significantly decreased against *E. coli* but unaffected against *S. aureus*. These results are in good agreement with the IgA, LTF and LYZ losses documented after these treatments. Especially for LTF, heat treatments have been shown to reduce its iron–binding capacity [46], which may have contributed to the significant increase in the *E. coli* growth rate in all heat–treated DHM samples. Other studies investigating the effect of heat treatment on the DHM bacteriostatic capacity, found a similar decrease after HoP [5, 47, 48]. The heat–induced denaturation and

aggregation during HoP could further explain the loss of protein functionality [13, 49]. In contrast to our findings, Silvestre et al (2008) reported a higher decrease in the DHM bacteriostatic capacity after pasteurization at 75°C for 15 sec than after 63°C for 30 min [50]. These differences may be attributed to the different pasteurization designs and the different bacterial strains used. Taken together, treatments with longer processing times, such as HoP, have a significantly larger impact on the DHM bacteriostatic capacity.

4.4.2. Effects of processing times and temperatures on BSSL level and activity

Since BSSL is a heat labile enzyme that starts inactivating at temperatures of 45°C [30], the great loss documented after all thermal treatments was to be expected. Wardell et al., (1984) showed that even a short exposure at 55°C can inactivate the enzyme, which explains the <20% retention that was documented after HTST. BSSL level and activity were almost completely abolished after HoP, as previously reported [10, 19, 51, 52]. FH, T99, HoP_r and T74 affected BSSL in a similar manner, independently of the different heating up and holding times applied. When comparing HTST to HoP, the significantly higher BSSL activity detected after HTST, may be attributed to the considerably shorter DHM exposure time over 55°C (33 sec versus 44 min). Similar observations have been previously documented [19, 52]. These findings suggest that non–thermal processing methods, such as ultraviolet–C irradiation or high–pressure processing, may offer substantially better results [51, 53].

4.5. Conclusion

Heat treatments, such as HTST, with considerably shorter processing times than the currently recommended HoP, were found to improve the retention of key DHM bioactive components. Our findings suggest that both reduced heating up and holding times are an essential factor for pasteurization optimization, as well as increasing the DHM quality. Since the recipients of pasteurized DHM are high–risk infants, these outcomes are of crucial importance. The pasteurization treatments used in this study are all assumed to lead to a safe product based on the inactivation of alkaline phosphatase, but whether inactivation of spore–forming pathogens such as B. cereus is achieved with such treatments is yet unknown. Moreover, considering that all thermal treatments caused a major reduction both in BSSL levels and activity and in bacteriostatic capacity, future studies should additionally investigate the effects of non–thermal methods on these components.

Conflict of Interest

The authors declare no conflicts of interests. JG is the founder and director of the Dutch National Human Milk Bank and member of the Dutch National Health Council. BS is as Science Director of Human Milk Research & Analytical Sciences an employee of Danone Nutricia Research, Utrecht, The Netherlands.

Acknowledgements

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Supplementary Appendix

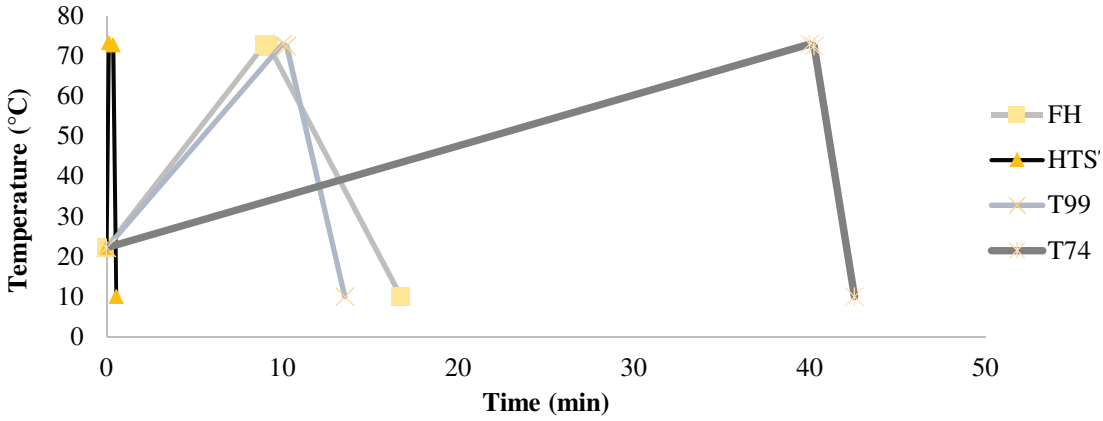

Figure S1. Time–temperature profiles of pasteurization methods at 72°C for 15 sec. HTST, FH, T99 and T74 stand for high–temperature short–time, flash heating, high–temperature short–time with a thermomixer preheated at 99°C and high–temperature short–time with a thermomixer preheated at 74°C, respectively.

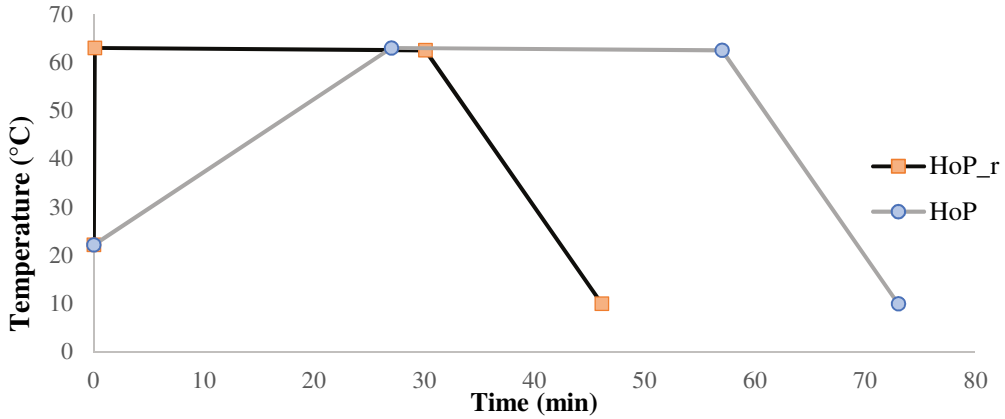

Figure S2. Time–temperature profiles of pasteurization methods at 62.5°C for 30 min. HoP and HoP_r stand for holder pasteurization and holder pasteurization with rapid heating up–times, respectively

Chapter 5

Effects of High–Pressure Processing, UV–C irradiation and thermoultrasonication on donor human milk safety and quality

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Abstract

Holder pasteurization (HoP) is the current recommended treatment for donor human milk. Although this method inactivates microbial contaminants, it also negatively affects various milk components. High–pressure processing (HPP, 400, 500 and 600 MPa), ultraviolet–C irradiation (UV–C, 2430, 3645 and 4863 J/L) and thermoultrasonication (TUS, 1080 kJ/L and 1620 kJ/L) were investigated as alternatives to thermal pasteurization (HoP). We assessed the effects of these methods on microbiological safety, and on concentration and functionality of immunoglobulin A, lactoferrin, lysozyme and bile salt–stimulated lipase, with LC–MS/MS–based proteomics and activity assays. HoP, HPP, TUS and UV–C at 4863 J/L, achieved >5–$\log_{10}$ microbial reduction. Native protein levels and functionality showed the highest reduction following HoP, while no significant reduction was found after less intense HPP and all UV–C treatments. Immunoglobulin A, lactoferrin and lysozyme contents were also preserved after low intensity TUS, but bile salt–stimulated lipase activity was significantly reduced. This study demonstrated that HPP and UV–C may be considered as suitable alternatives to HoP, since they were able to ensure sufficient microbial inactivation while at the same time better preserving the bioactive components of donor human milk. In summary, our results provide valuable insights regarding the evaluation and selection of suitable processing methods for donor human milk treatment, which may replace HoP in the future.

Keywords: Donor human milk; non–thermal processing; bacteria inactivation; bacteriostatic properties; proteomics; antimicrobial proteins

5.1. Introduction

Human milk (HM) is universally identified as the normative standard for infant nutrition, due to its unique nutritional composition and bioactive components such as immunoactive proteins, hormones, and growth factors, that facilitate proper infant growth and development [1]. An essential component of HM known for its bioactive function is the HM proteome, which is comprised of a wide array of proteins, glycoproteins, enzymes, and endogenous peptides [2]. For example, HM exerts bacteriostatic activity, a function largely ascribed to the presence of bioactive proteins, such as immunoglobulin A (IgA), lactoferrin (LTF) and lysozyme (LYZ), due to their high abundance in HM [3, 4]. More specifically, IgA protects the infant from invasive pathogens, LTF inhibits the growth of iron–dependent pathogens and LYZ lyses the proteoglycan matrix of the cell walls in Gram–positive bacteria. In addition, a synergistic effect of LYZ and LTF is suggested against Gram–negative bacteria [4–6].

In case mother's own milk is unavailable, donor human milk (DHM) is the best alternative and it should be provided by established human milk banks that enforce all necessary actions to guarantee its safety [7, 8]. Human milk banking guidelines recommend holder pasteurization (HoP) for the elimination of possible life–threatening pathogens in DHM [9]. This method is performed by heating DHM for 30 min at a temperature of 62.5 °C, followed by a rapid cooling down to <10°C [10].

Even though HoP achieves the 5–$\log_{10}$ reduction of vegetative bacterial cells required by all human milk banking guidelines, it also leads to the degradation of key DHM bioactive components [9]. After HoP, a significant reduction has been reported in the concentration and

activity of IgA, LTF and LYZ, as well as in several enzymes, hormones, cytokines and growth factors [10, 11]. In addition, HoP completely inactivates bile salt–stimulated lipase (BSSL), a heat–labile enzyme that facilitates fat absorption and enhances lipid metabolism [11]. It is thus possible that BSSL inactivation through HoP may be the cause of the reported lower growth rates of preterm infants fed with HoP–treated DHM, compared with the ones fed mother's own milk [12]. To overcome the disadvantages of this treatment, novel methods such as high–pressure processing (HPP), ultraviolet–C irradiation (UV–C) and thermoultrasonication (TUS) have been proposed as promising non–thermal alternatives to HoP [9, 13].

HPP is a mild food preservation method commonly applied in the food industry to guarantee the food safety of a product by microbial inactivation due to the high hydrostatic pressure [14, 15]. UV irradiation is a non–thermal disinfection method, especially at wavelengths between 200 and 280 nm (UV–C) [16]. This method effectively inactivates microbial contaminants by disrupting DNA transcription and replication, ultimately leading to cell death [16, 17]. Ultrasonication (20–100 kHz) is a food preservation method that involves microbubble formation and their rapid collapse though inertial cavitation. The shock waves that are produced from this process, as well as the chemical changes induced by it, ultimately lead to bacterial cell death [9, 18]. TUS, the process where ultrasonication is combined with mild heating, is considered more effective in microbial inactivation than ultrasonication alone [19]. In addition to bacterial inactivation, all the aforementioned methods are able to batch process human milk, as would be required when applying these methodologies in a human milk bank.

When applied to DHM, these methods have shown promising results with regards to microbial inactivation and retention of DHM bioactive components[9, 13, 20]. However, the number of studies evaluating UV–C or TUS as possible alternatives to HoP is still quite limited, while a large number of different pressure, time and temperature combinations have been applied for HPP to DHM, making direct comparison of those studies complicated [9].

The aim of this study was first to assess whether HPP, UV–C and TUS can achieve a 5–$\log_{10}$ bacterial reduction as found following HoP, which is a primary requirement for use of DHM. Secondly, we aimed to evaluate the effects of these methods on the DHM proteome in order to get a full overview of the changes caused, as well as on the concentration and bioactivity of IgA, LTF, LYZ and BSSL, and compare them with HoP.

5.2. Materials and methods

5.2.1. Milk samples

The HM samples used in this study were provided by the Dutch Human Milk Bank (located at Amsterdam UMC, Amsterdam, The Netherlands). Donor screening and milk collection was performed according to standardized procedures that comply to international guidelines [10]. All donors signed informed consent before recruitment. Milk expression, collection and transportation to the Dutch Human Milk Bank was performed as previously described [21]. The samples were transported frozen (–20˚C) to the human milk bank and were stored frozen at the same temperature, for a maximum of three months, until further processed. Before analysis, each donated sample was thawed overnight in a refrigerator at 4 °C. Once thawed, the native microflora of the samples was assessed by pour or surface–plating of undiluted DHM (1mL or 0.1mL, respectively) in duplicate onto selective media (VRBGA, violet red bile glucose agar,

CM0107B and MSA, mannitol salt agar, CM0085B, Thermo Fisher Scientific, Massachusetts, USA) and non–selective media (PCA, Plate Count Agar, CM0325, Thermo Fisher Scientific, Massachusetts, USA) followed by an incubation at 37 ˚C for 24–48 hours, while the remaining amount of each sample was again placed in the freezer (–20˚C). Bacterial numbers were determined by colony counting (CFU/mL), and the samples with 1 $\log_{10}$ CFU/mL or less were selected. Next, the samples were again thawed overnight at 4 °C, and milk from four different donors was pooled to ensure sufficient amounts of DHM for all treatments. One pool (four donors, 1000 mL) was used to evaluate the inactivation of the selected bacterial strains and another pool (four donors, 1000 mL) was used to evaluate the DHM proteome, the total protein content, the BSSL activity and the bacteriostatic properties. Aliquots for each treatment (HPP, UV–C, TUS, HoP) and for the untreated milk (UN) that served as control were then created. After all treatments, the samples were cooled in an ice–water bath and all analyses were performed immediately. The analyses were performed as two independent experiments (biological replicates), in duplicate (technical replicates). The experimental approach used in this study is presented in **Figure 1.**

5.2.2. Treatments

5.2.2.1. High-Pressure Processing

High pressure treatment was carried out in a pilot-scale equipment, custom made by Resato (1.6L, Resato, Roden, The Netherlands). The computer-controlled pressure build up was ~30 MPa/s. The samples were subjected to three different pressures for various holding times; 400 MPa for 5, 10, and 30 min, 500 MPa for 1.5, 2 x 1.5, 3, and 5 min, 600 MPa for 1.5, 2 x 1.5, 3, and 5 min. For the treatments "2 x 1.5 min", the process of pressure build up, holding time of 1.5 min and pressure release was carried out twice. This was done based on previous findings which demonstrated that two pressure treatments with 1.5 min holding time were more effective in microbial inactivation than one pressure treatment of 3 or 4 min at the same pressure [22]. DHM samples (10 mL, 4 °C) were packed into sterile pouches made of polyethylene. Two to four small sample pouches were packed in a larger pouch which was subsequently vacuumized at 95% vacuum. The larger pouches were then taped in a cylindrical holder that was placed in a sample holder as described previously [14]. Samples were not preheated and tap water of 10 °C was used at the start of the treatment as medium. The temperature increase during HPP treatment was described as follows [14]: $T_{increase}/100\ MPa = 0.026 \times T_{initial} + 2.26$, leading to a maximum sample temperature of around 18°C (after 600 MPa).

5.2.2.2. Ultraviolet–C irradiation treatment

The UV–C system was based on published literature [16], where a UV–C lamp (TUV PL–S 5W, UV–C radiation 1.1 W, Philips, the Netherlands) was placed diagonally in a sterile beaker glass filled with 140 mL DHM. During treatment, the milk was stirred with a sterile 4x20 mm stirring rod at 500 rpm (IKA RH 2, Staufen, Germany). Samples (20 mL, 4 °C) were taken at three different time points and were aliquoted for further analysis. The samples were exposed to three different UV–C dosages; 2430 J/L, 3645 J/L and 4863 J/L. The time needed to reach these dosages (5.15, 6.63 and 7.36 min, respectively) was calculated according to:

$Dosage\ (\frac{J}{L}) = \frac{Time\ (s) \times UV-C\ Power(W)}{Volume(L)}$ [16]. The temperature was controlled during the whole process with a temperature data logger (RS PRO 1384, RS Components B.V., The Netherlands) and a maximum increase of 3°C was documented after a treatment of 4863 J/L. This set–up was used in order to overcome the limited penetration of UV–C in milk (absorption coefficient of 300 cm^{-1} at 254 nm), by applying a turbulent flow [16].

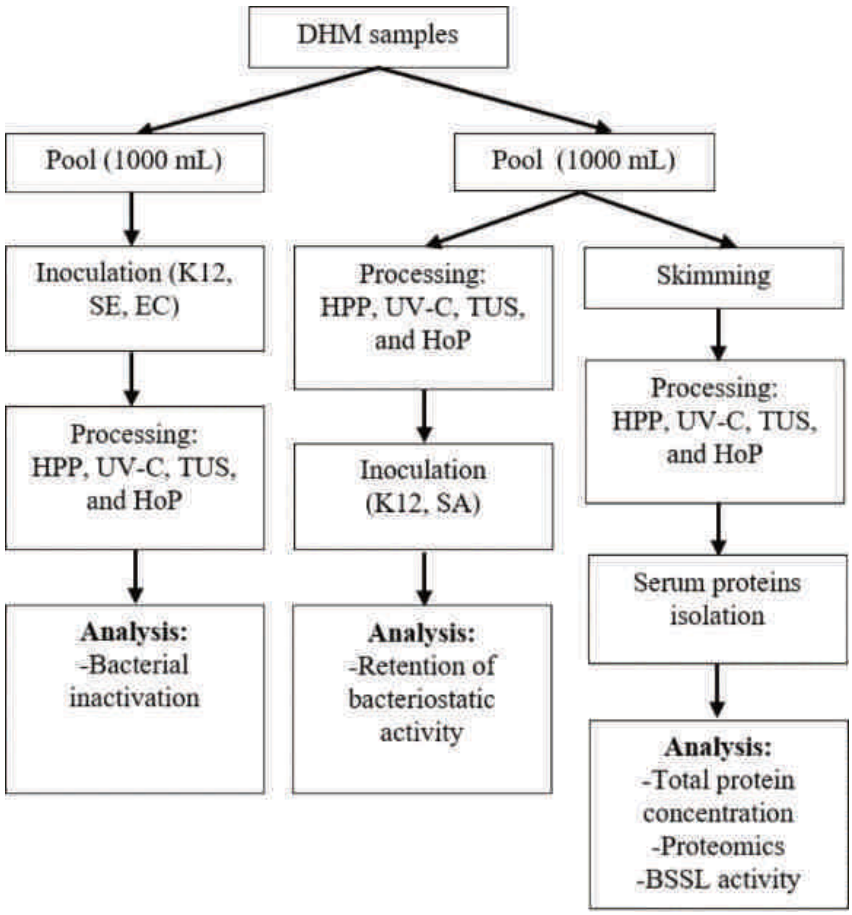

Figure 1. Schematic representation of the experimental approach used. K12, SE and EC stand for *E. coli* K12, *S. epidermidis* and *E. cloacae*, respectively. Two independent experiments (biological replicates) were performed while all analyses were performed in technical duplicate for each sample.

5.2.2.3. Thermoultrasonication

A sonifier (Branson 450 Digital Sonifier®, Branson Ultrasonics Corporation, Connecticut, USA) with a horn frequency of 20 kHz was outfitted with a sound enclosure (Branson Emerson Technologies, GmbH & Co, Germany), a microtip probe (length: 60 mm, diameter: 10 mm), and a circulating water bath. Samples (20 mL, 4 °C) were placed into a sterile 80 mL glass beaker that was surrounded by circulating water of 40 °C. The sonifier was operated in pulse mode, with a continuous pulse of 59.9 s followed by a short pause of 30 s. The samples were treated for 9 min (excluding pause time) at 40W (1080 kJ/L, 38% amplitude), for 6 min at 60W (1080 kJ/L, 58% amplitude) or for 9 min at 60 W (1620 kJ/L, 58% amplitude). The energy density (KJ/L) was calculated as power (W) x treatment time (sec)/volume (mL) [23]. The temperature of the samples was recorded by a temperature data logger (RS PRO 1384, RS

Components B.V., The Netherlands). The maximum temperature increase was 20 °C (maximum sample temperature, 59 °C), after 9 min at 60 W.

5.2.2.4. Holder Pasteurization

DHM (30 mL) was placed into a Greiner tube (50 mL) and was heated at 62.5 °C for 30 min, in a shaking water bath (150 rpm). The sample was cooled in an ice–water bath immediately after treatment, until a temperature 4 °C was reached. The time required for the temperature of the sample to reach the pasteurization temperature (62.5 °C) was 25 min, while the cooling down time to 4°C was 15 min. A temperature data logger RS PRO 1384 (RS Components B.V., The Netherlands) was used to monitor the temperatures during the whole process.

5.2.3. Bacterial inactivation

Bacterial species were selected based on their clinical relevance for DHM. Fresh cultures of *Enterobacter cloacae* (ATCC 13047, American Type Culture Collection, Manassas, USA), *Escherichia coli* K12 (DSM 498, Deutsche Sammlung von Mikroorganismen und Zellkulturen , Braunschweig, Germany) and *Staphylococcus epidermidis* (ATCC 14990, American Type Culture Collection, Manassas, USA) were prepared from frozen stocks in brain heart infusion broth (CM1135, Thermo Fisher Scientific, Massachusetts, USA) after an overnight incubation at 37 ˚C. The bacterial pellets that were obtained after centrifugation at 4000×g for 10 min (Microcentrifuge 5890R, Eppendorf, Hamburg, Germany), were subsequently inoculated into DHM samples at a final concentration of 10^8 CFU/mL and were then subjected to HPP, UV–C, TUS or HoP treatment. Treated and untreated samples were next plated in duplicate onto VRBGA (*E. coli*, *E. cloacae*) and MSA (*S. epidermidis*) and were incubated overnight at optimal growth conditions. Untreated but inoculated samples with the three strains served as reference to verify the starting microbial concentration and to calculate bacterial reduction. The reduction in bacterial numbers was determined by colony counting (CFU/mL), with a detection limit of 0 $\log_{10}$ CFU/mL (*E. coli* and *E. cloacae* counts) and 1 $\log_{10}$ CFU/mL (*S. aureus* counts). Since the HPP unit used in this study is located in a food safe environment, inoculation with pathogenic strains was prohibited. Therefore, all the strains used in this study were biosafety level 1 strains. In addition, bacteria inactivation after HPP was tested only with the *E. cloacae* and *S. epidermidis* strains.

5.2.4. Milk serum preparation and total protein content

To obtain the native milk serum proteins, after all treatments, caseins and denatured proteins were removed. To do so, untreated samples (520 mL) were first centrifuged at 6500×g for 30 min at 4 ˚C (with rotor 16.250, Avanti Centrifuge J–26 XP, Beckman Coulter, USA) to remove the fat. The skimmed samples were then treated with all methods as described above (sections 2.2.1–2.2.4), apart from one sample that remained untreated (control). Next, the pH of the skimmed samples was adjusted to 4.6 by the addition of 1 mol/L HCl under stirring, to precipitate the caseins and the denatured serum proteins [24]. The samples were left for 30 min at 4 °C to equilibrate and were subsequently ultracentrifuged at 100,000×g for 90 min at 30 °C (Optima L–80, Beckman Coulter, USA). Finally, the casein pellet was discarded, and the supernatant containing the native serum proteins was collected. The total native protein content

was then assessed using the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions.

5.2.5. Protein quantification and identification by liquid chromatography with tandem mass spectrometry (LC–MS/MS)

5.2.5.1. Filter aided sample preparation (FASP)

The FASP method was carried out as previously reported [25, 26]. Briefly, milk serum samples were diluted with 100mM Tris (pH 8.0) to a protein concentration of 1.0 µg/µL. The next steps included; DDT reduction (10 µL, 0.15 M), alkylation with 136 µL urea (8 M) in 100 mM Tris/HCl (0.1 M, pH 8.0) and 20 µL of acrylamide (0.2 M), placing the samples into ethanol washed Pall 3K omega filters (10–20 kDa cut off, OD003C34, Pall corporation, Port Washington NY, USA) and centrifuging them at 14000×g for 30 min, adding 110 µL 50 mmol/L NH_4HCO_3 to the filters and centrifuging them again (14000×g for 30 min). The samples were then digested with 1 µL trypsin (0.5 ug/ul sequencing grade) in 100 µL of NH_4HCO_3 (0.05 M) and after an overnight incubation, they were centrifuged for 30 min at 14000×g. After the addition of 100 µL 1 mL/L HCOOH in water on the filters, another centrifugation followed (14000×g for 30 min). Finally, 3 µL of TFA (10% v/v) was added to the filtrate to adjust the pH of the samples to 3. All samples were stored at –20 °C prior to LC–MS/MS analysis.

5.2.5.2. LC–MS/MS analysis

The LC–MS/MS analysis was performed as previously described [27]. Briefly, the samples (5 µL) were injected onto a 0.10 x 250 mm ReproSil–Pur 120 C18–AQ 1.9 µm beads analytical column that was prepared in house, using pressure of 800 bar. The peptides were then eluted at a flow of 0.5 µL/min with an acetonitrile gradient. The gradient elution increased from 9% to 34% acetonitrile in water with 1 ml/L formic acid in 50 min. Next, an electrospray potential of 3.5 kV was applied straight to the eluent, through a needle that was equipped to the P777 Upchurch micro cross waste line. Using a Q Exactive HF–X quadrupole–Orbitrap mass spectrometer (Thermo Electron, San Jose, CA, USA), full scan Fourier Transform MS in positive mode between m/z 380 and 1400 were measured. MS/MS scans of the most abundant multiply–charged peaks were recorded in data–dependent mode. The obtained MS/MS data was analyzed using the Andromeda search engine of the MaxQuant software (v1.6.3.4). The Uniprot human protein database was used, together with a database containing the sequences of common contaminants [28]. Protein identification and quantification was performed as previously described [29, 30]. To calculate the false discovery rate (FDR), MaxQuant created a decoy database of reversed sequences. The FDR cut off used was 0.01. The required peptide length was set to at least seven amino acids, with a maximum of 2 missed cleavages allowed. Protein modifications were set for propionamide (C) (fixed) and oxidation (M) (variable). Contaminants (e.g. keratins, trypsin) were removed from the set of identified proteins, as well as the proteins that were detected in less than half of our samples.

5.2.6. BSSL activity

BSSL activity was determined according to Krewinkel et al. (2016) with minor modifications [31]. This fluorometric assay allows the determination of lipase activity in DHM by utilizing

the synthetic substrates 4–methylumbelliferyl butyrate (4–MUB) and 4–methylumbelliferyl laurate (4–MUL). Milk samples were first skimmed as described in section 2.4 and were then preincubated at 40 °C for 3 min, under shaking (800 rpm) in a ThermoMixer (SmartBlock 1.5 ml, Eppendorf, Hamburg, Germany). The conversion of the added substrate was stopped by the addition of a stop solution containing GuHCl (8 M) and HCl (1 M) in water. Next, a neutralizing solution with Bis–tris (1M), NaOH (0.85 M) and EDTA (0.25 M) in water was added to clarify the samples. The fluorescence was then measured with a fluorimeter (excitation 355 nm, emission 460 nm).

5.2.7. DHM bacteriostatic capacity

To evaluate the effect of processing on the bacteriostatic capacity of DHM, the growth rate of *Escherichia coli* and *Staphylococcus aureus*, which are known to be sensitive to these proteins was characterized [6, 32, 33]. Bacterial pellets of *E. coli* K12 (DSM 498) and *S. aureus* (ATCC6538, American Type Culture Collection, Manassas, USA) were prepared as described in section 2.3 and were dissolved in peptone physiological salt solutions (PFZ; Tritium Microbiology, The Netherlands). After determining the optical density (OD) with a spectrophotometer (Cary 50 UV–Visible Spectrophotometer, Agilent Technologies, USA) for bacterial culture standardization [34, 35], *E. coli* and *S. aureus* cultures were diluted and inoculated into untreated samples and samples that were previously treated with HPP, UV–C, TUS and HoP, to a final concentration of around 10^3 colony forming units (CFU)/mL. This inoculation level was selected because higher levels may overcome the ability of the milk to inhibit the growth of *E. coli* and *S. aureus* [36]. Next, the samples inoculated with *E. coli* and *S. aureus* were incubated at 37 °C for 2 h and 4 h, respectively. All samples were then plated in duplicate onto VRBGA (selective for *E. coli*) and MSA (selective for *S. aureus*) and the plates were subsequently incubated overnight at 37 °C. The amount of inoculated DHM sample plated was 1 mL and 0.1 mL for VRBGA and MSA, respectively. The bacterial concentrations were then determined by colony counting (CFU/mL). The growth rate per hour was calculated as $ln(\frac{N_t}{N_0})/\,t$, were N_t = bacterial counts after 2 h or 4 h incubation, N_0 = bacterial counts immediately after inoculation and t = incubation time.

5.2.8. Data Analysis

Data analysis and visualization were performed using GraphPad Prism software 8.0 (GraphPad Inc., La Jolla, CA). For multiple comparisons of means and to determine significant differences among the treatments, ANOVA and Tukey's HSD for post–hoc testing were performed. Protein retentions (% compared to untreated) were calculated as the ratio of the concentration after each treatment to the concentration of untreated samples, multiplied by 100. Perseus software v.1.6.2.1 was used to analyze the intensity based absolute quantitation (iBAQ) values that were determined by MaxQuant. These values refer to the sum of all peptide peak intensities divided by the number of theoretically generated tryptic peptides and are considered as a good indicator for the absolute protein concentration [30]. To indicate significant differences in the DHM proteome after the different treatments, student's t–tests were performed in Perseus after imputation of missing values, using permutation–based false discovery rate (FDR) correction. The cluster analysis was performed and visualized with the circos.heatmap package in R version

4.1.2 [37] on the imputed log_{10} scaled IBAQ values. Pearson correlations were also calculated to determine the relationship between the bacterial growth rate and the retention of IgA, LTF and LYZ. A correlation matrix was created using R version 3.4.0 [37]. Significant differences in all analyses were indicated by a p–value <0.05. Two independent experiments (biological replicates) were conducted and all analyses were performed in technical duplicates. Data are presented as mean ± standard deviation of the two independent experiments.

5.3. Results

5.3.1. Bacterial inactivation

The bacterial count reductions after HPP, UV–C, TUS and HoP are presented in **Table 1**. A >7.8–log_{10} inactivation of *E. cloacae* and *S. epidermidis* was obtained after all the different HPP conditions tested. The same inactivation was obtained after HoP for all tested bacterial strains. A UV–C dosage of 4863 J/L was the only UV–C dosage effective in causing a >5–log_{10} reduction of *E. cloacae, E. coli* K12 and *S. epidermidis* counts. All TUS conditions tested were able to achieve a >5–log_{10} reduction for all tested bacterial strains.

Table 1. Reduction in bacterial counts in HoP, HPP, UV–C and TUS treated DHM samples. The results are presented as mean ± standard deviation of two independent experiments and all analyses were performed in technical duplicate.

Methods	**Parameters**	**Log_{10} reduction (CFU/mL), mean ±SD**		
		Enterobacter cloacae	*Staphylococcus epidermidis*	*E. coli K12*
HoP	>7.8 (below the detection limit)			
HPP	400 MPa, 5–30 min	>7.8 (below the detection limit)		ND*
	500 MPa, 1.5–5 min			
	600 MPa, 1.5–5min			
UV–C	2430 J/L	4.25 ± 0.1	5.00 ± 0.3	4. 36 ± 0.1
	3645 J/L	4.64 ± 0.1	5.95 ± 0.1	5.30 ± 0.3
	4863 J/L	5.78 ± 0.2	6.95 ± 0.4	6.92 ± 0.1
Thermoultrasonication	40 W for 9 min	6.31 ± 0.4	6.07 ± 0.3	6.40 ± 0.1
	60 W for 6 min	6.63 ± 0.6	6.50 ± 0.5	6.73 ± 0.3
	60 W for 9 min	6.52 ± 0.5	6.21 ± 0.2	6.79 ± 0.4

*ND: Not determined. The detection limit was 0 log_{10} CFU/mL for *E. coli* and *E. cloacae* and 1 log_{10} CFU/mL for *S. aureus*.

5.3.2. *Protein damage*

5.3.2.1. Native milk serum protein concentration

The total native milk serum protein concentration after HPP, UV–C, TUS and HoP is shown in **Figure 2**. When compared to the untreated samples, a significant decrease in protein concentration was observed only after HoP (p<0.05).

5.3.2.2. Effects of processing on the DHM proteome

To further evaluate the effect of the different treatments on the native milk serum proteins, a detailed characterization of the DHM proteome was obtained, by means of LC–MS/MS. Next, a clustered heat map based on the obtained iBAQ values was created, for visualization of the protein profile of the different treated DHM samples (**Figure 3**). Samples with similar protein patterns are clustered together. The samples formed two main clusters; one that consists of the untreated, the HPP and the UV–C samples and one that includes the TUS and HoP samples. This clustering pattern indicates that HoP and TUS affect the DHM proteome the most, and similarly. In addition, the separation of the HPP treatments with the highest intensities (600MPa for 3 and 5 min) in the cluster with the other samples suggests that these most intense HPP treatments have a larger effect on the proteome compare to the less intense HPP and UV/C treatments.

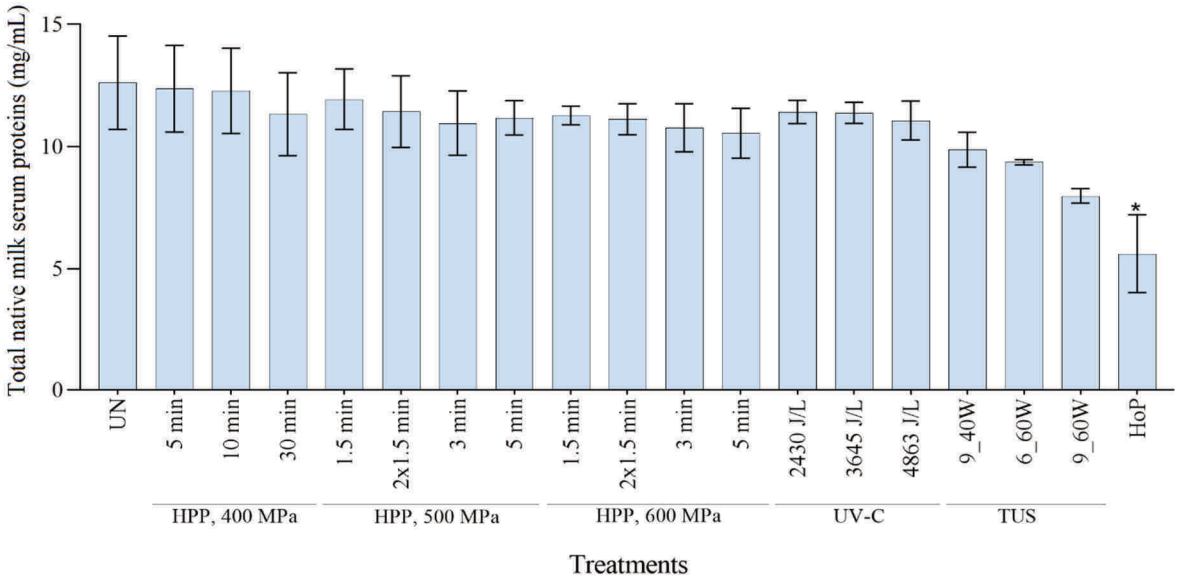

Figure 2. Native milk serum protein concentration as determined with a BCA assay. The results are presented as mean ± standard deviation of two independent experiments and all analyses were performed in technical duplicate.*Indicates significant differences to untreated samples ($p<0.05$).

5.3.2.3. Retention of IgA, LTF and LYZ after processing

Of all the proteins analyzed by LC–MS/MS as shown in **Figure 3**, IgA, LTF and LYZ are of importance due to their bacteriostatic activity. The retention values of the three proteins, as calculated from the LC–MS/MS data, were significantly reduced after HoP ($p<0.05$), with only 40%, 22% and 44% of IgA, LTF and LYZ levels being retained after HoP, respectively (**Figure 4**). At the same time, none of the HPP treatments tested caused a significant reduction in LTF and LYZ levels. Furthermore, no IgA losses occurred after HPP at 400 and 500 MPa, regardless of the treatment time. When the pressure intensity increased (600 MPa), a treatment of 3 min caused a major decrease (55% IgA retention), although statistically non–significant, while a treatment of 5 min caused a statistically significant decrease (47% IgA retention). None of the applied UV–C dosages caused a significant reduction on the levels of the three proteins. The

retentions of the three studied proteins showed a decreasing tendency with increasing TUS intensity and exposure time. After 6 min at 60 W, LYZ levels were significantly reduced, while after 9 min at 60 W, all three proteins were significantly reduced ($p<0.05$).

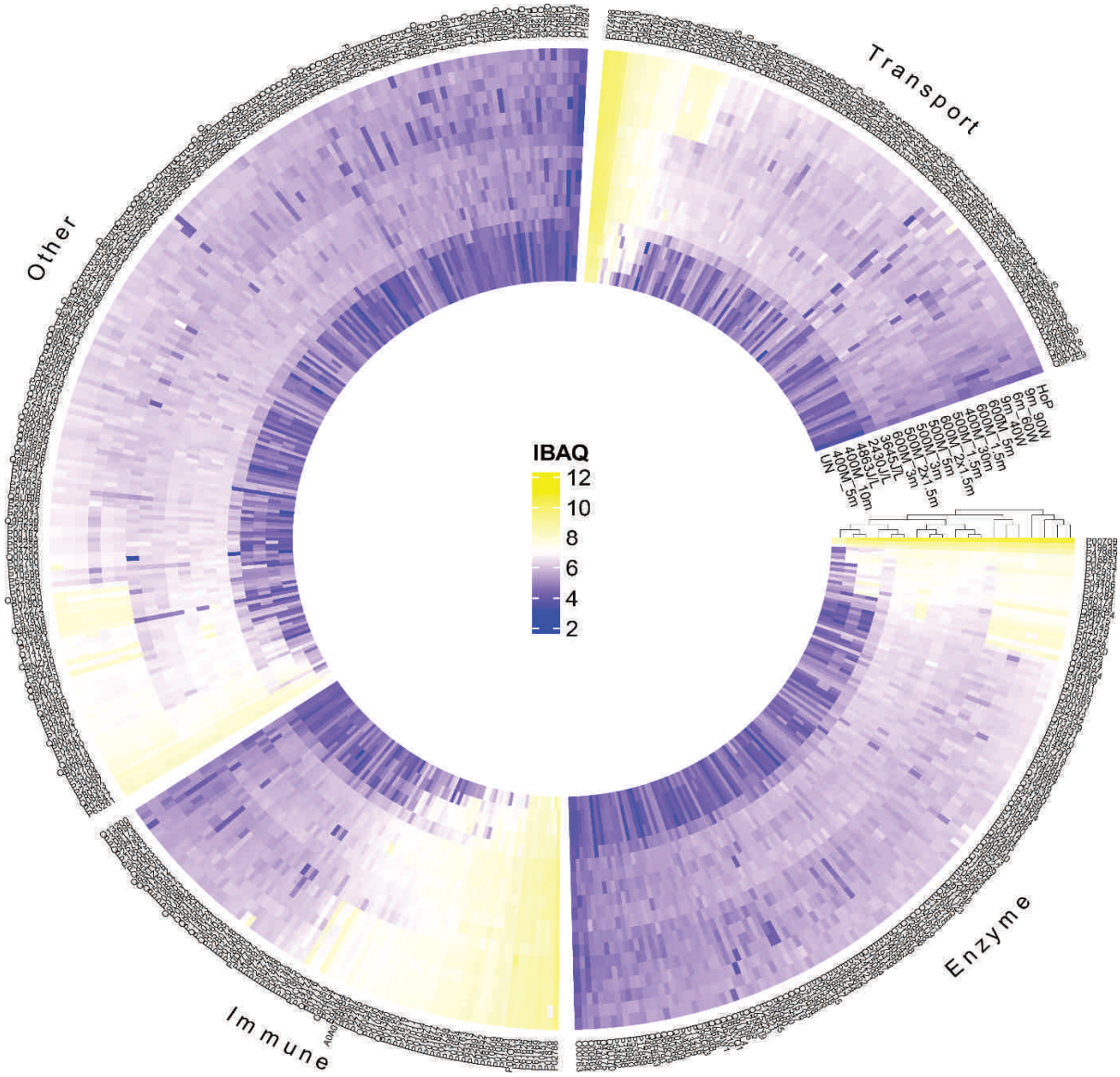

Figure 3. Hierarchical cluster analysis and heatmap showing the changes in the protein profile after HPP, UV–C, TUS and HoP, based on iBAQ values (log_{10} scale from 2 to 12 according to color bar). Proteins are labelled by their UniProt ID. Functional categories (enzyme, immune, transport, and other) were based on GO annotation of biological function. Two independent experiments (biological replicates) were performed while all analyses were performed in technical duplicate for each sample. UN represents the untreated values. HPP; 400 MPa for 5, 10, and 30 min, 500 MPa for 1.5, 2 x 1.5, 3, and 5 min, 600 MPa for 1.5, 2 x 1.5, 3, and 5 min, UV–C; 2430J/L, 3645J/L and 4863L/L,TUS; 9_40W, 6_60W and 9_60W.

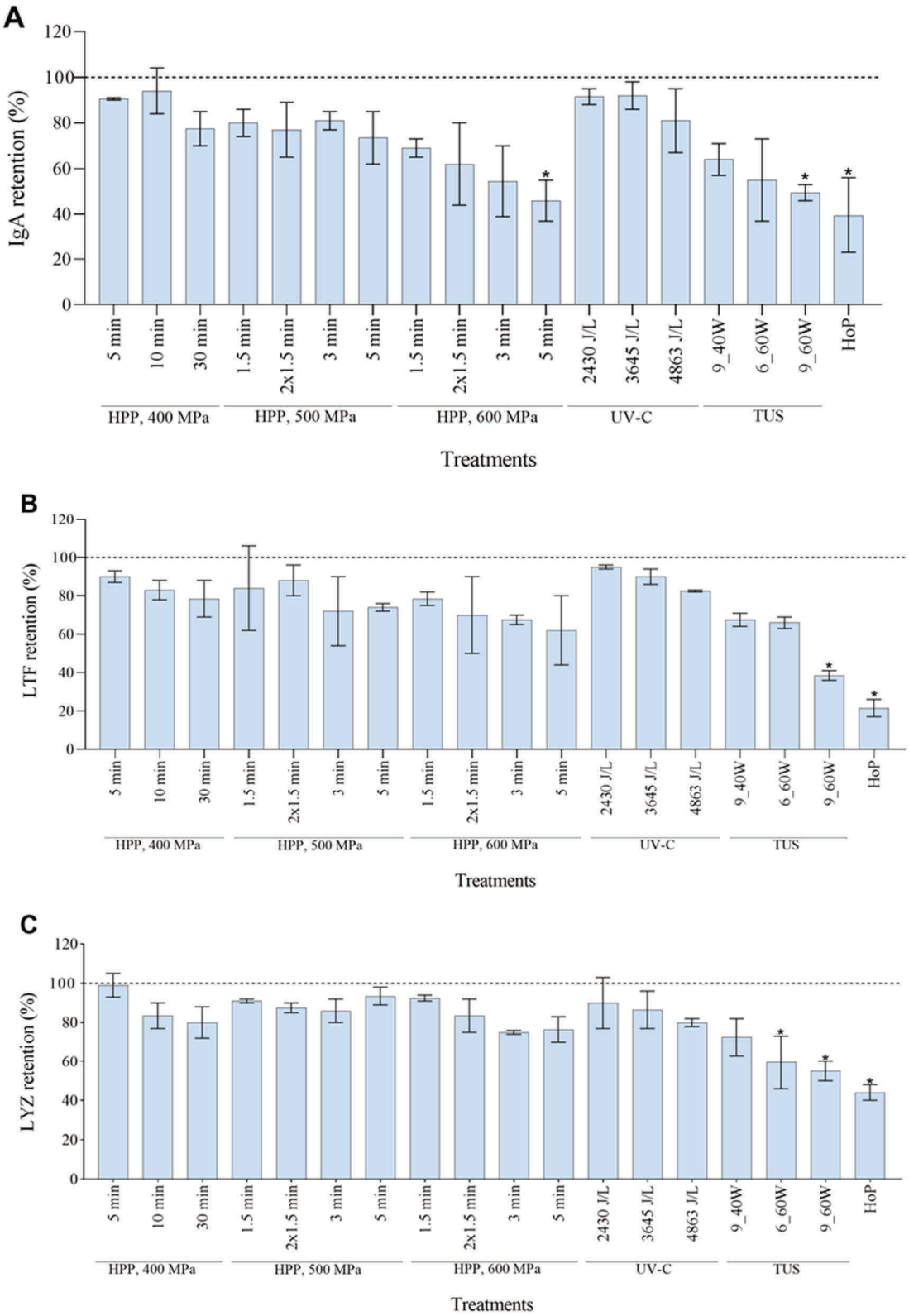

Figure 4. Effect of HPP, UV–C, TUS and HoP on the IgA (A), LTF (B) and LYZ (C) content. The retention values were calculated based on the iBAQ intensities obtained by LC–MS/MS analysis. Untreated values were set at 100% (dotted line). The results are presented as mean ± standard deviation of two independent experiments and all analyses were performed in technical duplicate.*Indicates significant differences to untreated samples ($p<0.05$).

5.3.2.4. BSSL retention after processing

To evaluate whether the different methods affected the BSSL levels and activity, we first determined the BSSL retention, based on the LC–MS/MS results. Then, a specific lipase activity assay was used (as described in section 2.6), and the percentage of BSSL activity retained after the different treatments was compared to the LC–MS/MS values (**Figure 5**). Although the majority of the values obtained by the activity assay were higher than the LC–MS/MS values, no significant differences between the retention values from both analytical methods were observed ($p>0.05$). After HoP, BSSL was almost completely diminished (LC–MS/MS, 3% and activity assay, 7%). On the contrary, the different HPP and UV–C treatments applied in this study did not lead to a significant decrease. However, BSSL retention decreased significantly after TUS, regardless of the intensity and the exposure time applied.

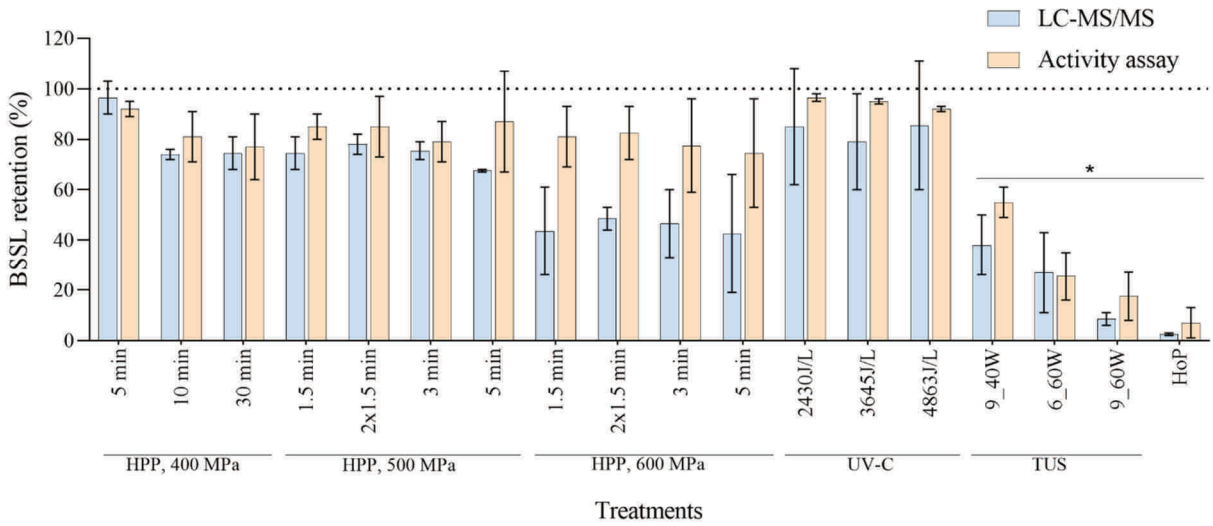

Figure 5. BSSL retention after HPP, UV–C, TUS and HoP, based on iBAQ intensities and a lipase activity assay. Untreated values were set at 100% (dotted line). The results are presented as mean ± standard deviation of two independent experiments and all analyses were performed in technical duplicate.*Indicates significant differences to untreated samples ($p<0.05$)

5.3.2.5. Retention of DHM bacteriostatic properties after processing

To evaluate whether the bacteriostatic capacity of DHM was retained after the different treatments, the growth rate of *S. aureus* and *E. coli* was characterized, in both untreated and treated samples (**Figure 6**). Untreated samples showed the lowest bacterial growth rate, thus the highest inhibition rate for both strains (1.6 ± 0.75 and 4.4 ± 0.04–fold per hour, for *S. aureus* and *E. coli*, respectively). In contrast, the highest bacterial growth rate was observed after HoP (3.9 ± 1.02 and 6.1 ± 0.70–fold per hour, for *S. aureus* and *E. coli*, respectively, $p<0.05$), which indicates a significant decrease in the DHM bacteriostatic capacity. When compared to the untreated samples, *E. coli* growth rate after HPP was not significantly different, while a significant increase in *S. aureus* growth rate was only observed at the highest intensities (3.0 ± 0.13 and 3.2 ± 0.40–fold per hour, after 600 MPa for 3 min and 600 MPa for 5min, respectively, $p<0.05$). After UV–C, as well as after TUS for 9 min at 40 W, bacterial growth rates were not significantly different to those of untreated samples. The growth rate of *S. aureus* was

significantly increased after 6 min at 60 W (3.0 ± 0.18–fold per hour), while after 9 min at the same intensity, the growth rates of both strains were significantly increased (*S. aureus*, 3.4 ± 0.69 and *E. coli*, 5.7 ± 0.66–fold per hour, p<0.05).

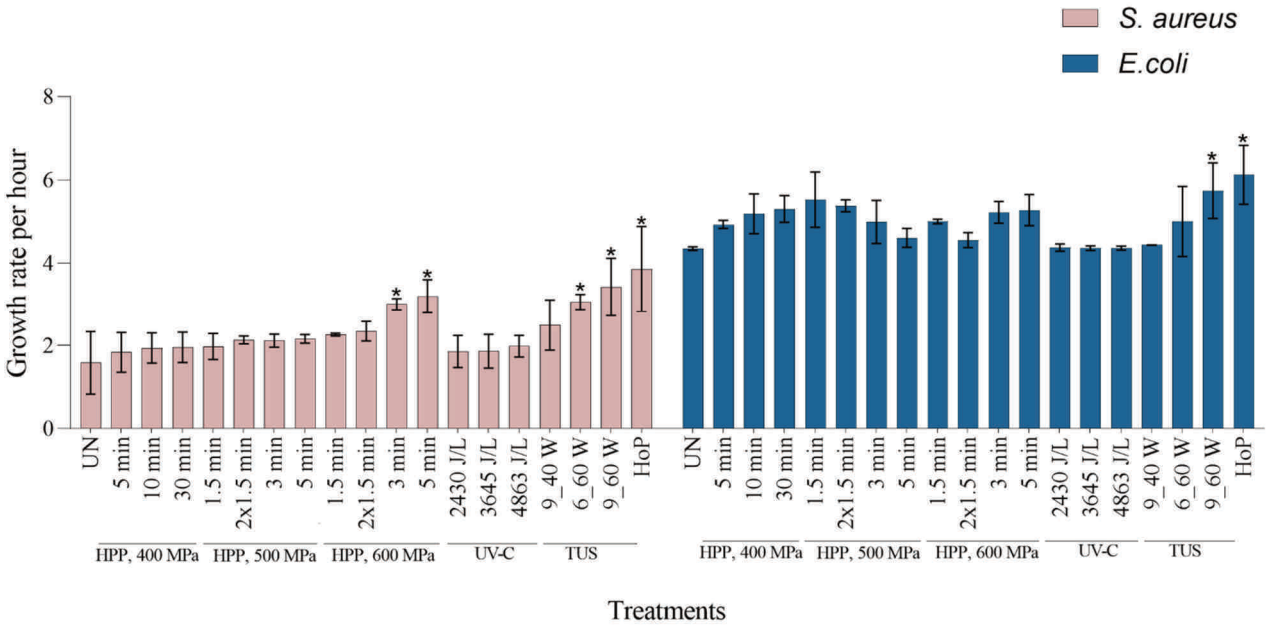

Figure 6. Growth rate per hour of S. aureus and E. coli in untreated, HPP, UV–C, TUS and HoP DHM samples. The results are presented as mean ± standard deviation of two independent experiments and all analyses were performed in technical duplicate.*Indicates significant differences to untreated samples (p<0.05).

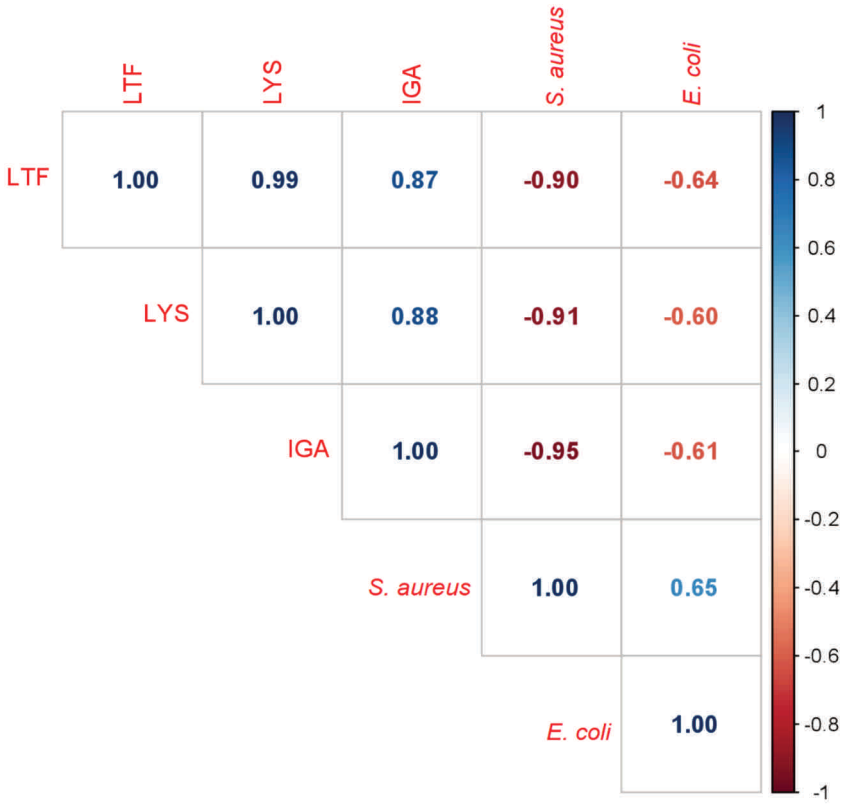

Figure 7. Correlation matrix of *S. aureus* and *E. coli* growth rates and IgA, LTF and LYZ iBAQ values. Each box contains an r value (Pearson correlation coefficient).

When the bacterial growth increased while the IgA, LTF and/or LYZ levels decreased, a negative correlation could be expected between the S. aureus and E. coli growth rate and the retentions of these three antimicrobial proteins. To confirm this bacteriostatic activity, a correlation matrix was created (**Figure 7**). **Figure 7** shows that S. aureus growth was strongly negatively correlated with the levels of these three antimicrobial proteins (IgA, r =–0.95, LTF, r =–0.90 and LYZ, r =–0.91, $p<0.05$). Although the correlation between the inhibition of E. coli growth and the concentrations of these proteins was weaker, it was still significant (IgA, r =–0.61, LTF, r =–0.64 and LYZ, r =–0.64, $p<0.05$).

5.4. Discussion

The present study demonstrates that the tested HPP and UV–C conditions preserved the levels and functionality of key DHM bioactive components better than HoP, while at the same time ensured sufficient microbial inactivation. Although the tested TUS conditions resulted in similar bacterial inactivation, this method was generally less efficient in retaining the DHM bioactive components.

5.4.1. Effects of processing on bacteria inactivation

All different HPP intensity–time combinations were able to achieve a reduction >7.8–$\log_{10}$ CFU/mL of *E. cloacae* and *S. epidermidis* counts (**Table 1**), even at the lowest condition of 400 MPa for 5 min. Similarly, coliform and *Enterobacteriaceae* counts were reduced to undetectable levels after 5 min at pressures of 400–600 MPa [38, 39]. Viazis et al. (2008) also found a ≥6–$\log_{10}$ reduction of *E. coli* and *S. aureus* counts after 400 MPa for 30 min, while an 8–$\log_{10}$ reduction of *Listeria monocytogenes* and *Streptococcus agalactiae* counts was already achieved after ≤4 min at the same intensity [40]. Although some *S. aureus* strains were found to be more pressure resistant, at higher pressures intensities significant reductions were achieved (500–600 MPa, or 400 MPa for 30 min for >5–$\log_{10}$ reduction) [40–42].

Only the highest UV–C dosage of 4863 J/L was capable of achieving a >5–$\log_{10}$ CFU/mL reduction of *E. cloacae*, *S. epidermidis* and *E. coli* K12 counts (**Table 1**). Christen et al. (2013b) showed similar reductions of *S. epidermidis*, *E. cloacae, Bacillus cereus*, and *E. coli* counts at 4863 J/L, and according to Li et al. (2017), the same dosage reduced DHM bacterial counts as effectively as HoP [16, 43]. Martysiak–Żurowska et al. (2017) reported a 5–$\log_{10}$ reduction of *S. aureus* and *E. coli* K12 counts already at much lower dosages (400 and 700 J/L, respectively) [17]. The differences in the experimental set up (e.g. actual UV–C output power, milk flow around lamp, milk compositional differences) used in these studies may account for the observed variation.

All TUS treatments tested in this study achieved a >6–$\log_{10}$ reduction of *E. cloacae*, *S. epidermidis* and *E. coli* K12 counts (**Table 1**). Similar results were observed by Czank et al. (2009), who found that the decimal reduction time of *S. epidermidis* and *E. coli* K12 was 1.74 and 2.08 min, respectively after TUS at 60 W and 45 °C [44].

In the current study, all HPP treatments, the highest UV–C dosage (4863 J/L), and all three TUS treatments were capable of reducing bacterial counts in DHM samples >5–$\log_{10}$, thus meeting the requirements of the human milk banking guidelines

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5.4.2. Effects of processing on the DHM proteome, IgA, LTF, LYZ, and BSSL levels and activity

In order to get a full overview of the impact of the different processing methods on the DHM proteome, the native milk serum protein levels were assessed by means of the BCA–assay and LC–MS/MS. With regard to HoP, our results (**Figure 2** and **Figure 3**) confirm the major decrease in native protein abundance [45]. Moreover, the proteomic analysis of the differently treated DHM samples showed that HoP affected the native serum protein levels the most (**Figure 3**), an outcome that supported the results of the BCA assay (**Figure 2**). Of all the treatments tested in this study, HoP caused the highest reduction in IgA, LTF and LYZ levels (**Figure 4**), which is consistent with the losses previously reported [11, 46]. As expected, the highest reduction in bacteriostatic capacity was also documented after HoP (**Figure 6**), which is in line with previous studies [6]. These results can be attributed to the thermally induced denaturation and aggregation during HoP, which caused a loss in the functionality of these bioactive components [44]. As BSSL is a heat–labile enzyme that inactivates at temperatures around 45 °C [47], the complete loss of BSSL that was observed in this study was to be expected [11, 13, 16, 48].

We showed that HPP treatments at intensities of 400, 500 MPa and of short duration (<3 min) at 600 MPa, preserve the levels of the three main antimicrobial proteins in DHM (**Figure 4**). Furthermore, our proteomic analysis showed that HPP treatments at these intensities had only minimal effects on the levels of the native milk serum protein levels, while more intense conditions showed a larger change in these levels. The enhanced denaturation observed after HPP at 600 MPa at longer treatment times might be explained by the fact that HPP can cause native conformation unfolding and formation of inter/intra protein complexes, where these changes may only be reversible at lower treatment intensities [39, 49, 50]. Moreover, increased protein denaturation has been observed at higher pressures and holding times, suggesting an effect of both pressure and holding time [51, 52]. Other studies have also reported significant reductions in IgA levels after HPP at 600 MPa for >2.5 min [39, 53–56][39, 53–56], while after treatment at 400–500 MPa, only 0–15% of IgA losses were documented [15, 38, 39]. Our data showed that treatments of 400, 500 and 600 MPa retained LTF levels within a range of 62–90%, as previously described [53, 57, 58]. In addition, none of the HPP treatments tested had an effect on LYZ levels, as well as on BSSL levels and activity, in line with previous studies [13, 15, 48, 55, 56, 59, 60]. Since pressure and temperature have a synergistic effect on protein denaturation, the low initial temperature (4 °C) and the limited temperature increase during HPP treatment (around 14 °C at the most intense pressure of 600 MPa), may have additionally contributed to the improved protein retentions observed [61–63].

As a non–thermal method, UV–C does not inactivate pathogens by thermally–induced protein denaturation and aggregation, but by DNA disruption, that often results from pyrimidine dimerization [43]. Hence, this method may effectively reduce bacterial counts in HM without causing detrimental losses of bioactive components [6, 43]. All UV–C treatments in our study preserved both the levels and the bioactivity of IgA, LTF and LYZ. In fact, the three antimicrobial proteins were retained within a range of 80–95% after UV–C treatment, while the bacteriostatic activity was similar to that observed in untreated HM (**Figure 4** and **6**). The clustering pattern observed for the three dosages additionally suggests that the changes occurring in the DHM proteome after UV–C are minimal (**Figure 3**). Christen et al. (2013a)

also reported retention of IgA, LTF and LYZ within a range of 75–95% and no loss of bacteriostatic activity, after treatments of the same intensity. As it is possible that UV–C induces protein photo–oxidation (direct or indirect) the authors speculated that the reductions (~25%) in LYZ levels at the highest dosage could be attributed to the fact that LYZ contains several amino acid residues that may absorb photons at this wavelength [6]. With respect to the BSSL levels and activity, none of the dosages in this study showed a significant reduction compared to untreated milk (**Figure 5**), supporting previous findings [16, 43, 60].

After TUS at the highest ultrasound power (60 W) for the longest exposure time (9 min), the IgA, LTF, LYZ and BSSL levels and bioactivity retained were comparable to those after HoP (**Figure4**). Similar reductions have been previously reported after 10 min at 60 W and 45 °C [64]. Treatments at 60 W for a shorter time (6 min) caused significant reductions in LYZ and BSSL levels and bioactivity, whereas at 40 W for 9 min, only BSSL was significantly reduced. Our findings suggest that at constant exposure times, higher ultrasound power will result in more protein damage. In addition, the differences observed when ultrasound energy was held constant (1080 kJ/L after 9 min at 40 W or 6 min at 60 W), suggest that higher ultrasound power rather than the longer exposure time may lead to more protein damage. The impact of those treatments on the DHM proteome was confirmed by the hierarchical clustering analysis, that showed a similar pattern of protein damage to HoP–treated DHM (**Figure 3**). These results could be further attributed to the temperature increase documented during such treatments, and to the denaturation that might be caused due to the shear effects generated during ultrasound cavitation [64, 65].

Specifically for BSSL, published reports have used both activity assays and quantification techniques (e.g. ELISA) to evaluate its retention [16, 66]. Since the loss of protein as measured through LC–MS/MS approaches or ELISA assays is not necessarily correlated to loss of function [67], we compared the retention of the BSSL levels (LC–MS/MS) and the BSSL activity (activity assay) after the different treatments. Our findings suggest that although the activity assay produced higher values than LC–MS/MS values, no significant differences were observed between the results of the two methods.

Lastly, the correlations in this study between native IgA, LTF and LYZ levels and the growth rate of bacteria sensitive to these proteins, suggest that these proteins may significantly contribute in limiting their growth (**Figure 7**). However, as DHM contains large numbers of antimicrobial components, the exact proportion of bacteriostatic activity attributed to those proteins is difficult to determine. These findings underline the importance of using complementary assays to determine protein levels and functionality, to accurately assess the effects of different processing methods. In this regard, many analytical techniques are available that can be used for future, more detailed characterization of such HM components and their functionality [27, 45, 68]. In addition, the outcomes of our study suggest that certain proteins may be more sensitive to specific non–thermal treatments than others, due to the different underlying mechanisms of these treatments. Our proteomic analysis, for example, showed that even though TUS and high HPP intensities both cause protein damage, they cluster separately (**Figure 3**), indicating that different underlying mechanisms lead to a different profile of resulting protein damage. In summary, HPP at 400 MPa, 500 MPa and 600 MPa for <3 min, as well as UV–C at 4863 J/L, may be promising alternatives to HoP, when considering the

sufficient microbial inactivation achieved and the improved outcomes on the preservation of important HM bioactive components.

5.5. Conclusion

Although HoP is the method currently recommended for DHM processing, the results of the current study indicate that non–thermal methods such as HPP and UV–C may offer improved retention of key DHM bioactive components, while at the same time effectively reduce bacterial contaminants. These findings are of particular importance in the context of providing DHM to high–risk infants. However, before full–scale implementation of these technologies in a human milk bank setting, additional studies are needed to investigate both viral inactivation and the clinical significance of this study's observations, especially with regards to growth rates and health status of infants fed DHM treated with HPP or UV–C.

Declaration of competing interest

The authors declare no other conflicts of interests. JbvG is the founder and director of the Dutch National Human Milk Bank and member of the Dutch National Health Council. B.S. is as Science Director of Human Milk Research & Analytical Sciences an employee of Danone Nutricia Research, Utrecht, The Netherlands.

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Chapter 6

Thermoultrasonication, ultraviolet–C irradiation, and high–pressure processing: Novel techniques to preserve insulin in donor human milk

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Abstract

Donor human milk (DHM) is recommended as the first alternative for preterm infants if their mother's own milk is not available or if the quantity is not sufficient. The most commonly used technique to eliminate microbial contaminants in DHM is holder pasteurization (HoP). However, the heating process during HoP partially destroys milk bioactive factors such as insulin. Therefore, innovative techniques have been developed as alternatives to HoP. The objective of this study was to determine the effect of HoP, high–temperature–short–time (HTST), thermoultrasonication (TUS), ultraviolet–C irradiation (UV–C), and high–pressure processing (HPP) on the insulin concentration in DHM.

Milk samples from 28 non–diabetic mothers were collected. The milk samples were aliquoted and either left untreated or treated with HoP (62.5°C; 30 min), HTST (72°C; 15 s), TUS (60 W; 6 min), UV–C (4863 J/L), or HPP (500 MPa; 5 min).

The mean insulin concentration in untreated milk was 79 ± 41 pmol/L. The mean ± SD insulin retention rate (%) was 71 ± 19 for HoP, 82 ± 20 for HTST, 101 ± 12 for TUS, 93 ± 7 for UV–C, and 106 ± 7 for HPP. The mean insulin concentration in milk treated with HoP was significantly lower compared to untreated milk (p=0.01).

TUS, UV–C, and HPP preserve insulin in DHM. The insulin concentration in DHM is affected to a larger extent by HoP than by HTST. These results indicate that TUS, UV–C and HPP may serve as alternatives to HoP.

Keywords: Trophic factor; breastfeeding; milk bank; preterm; pasteurization

6.1. Introduction

Pasteurized donor human milk (DHM) is recommended as the first alternative for preterm infants if their mother's own milk is not available or if the quantity is not sufficient [1]. Pasteurization of DHM ensures microbiological safety. The currently recommended method is holder pasteurization (HoP), which includes heating the milk for 30 min at 62.5°C [2]. However, this heating process partially destroys bioactive factors, thereby reducing the quality of DHM [2].

To improve the quality of DHM, innovative techniques, such as high–temperature–short–time (HTST), thermoultrasonication (TUS), ultraviolet–C irradiation (UV–C), and high–pressure processing (HPP) are currently under investigation as alternatives for HoP [2, 3]. In several studies, higher retention rates of various nutritional and bioactive factors (e.g., immunological components and enzymes) were achieved using these techniques compared to HoP, while pathogens were still adequately inactivated [2, 3]. The effect of these innovative techniques on milk hormones, such as insulin, has rarely been investigated [2, 3].

Milk insulin appears to be a key factor for optimal gastrointestinal development, given that this hormone stimulated intestinal maturation *in vitro* and in small–scale *in vivo* experiments [4, 5]. The insulin concentration is reported to be significantly reduced in DHM after HoP [2]. Therefore, the gastrointestinal development and clinical outcomes of preterm infants who are fed DHM may improve by innovative techniques that better preserve insulin in DHM.

As such, the objective of this study was to determine the effect of HoP, HTST, TUS, UV–C, and HPP on the insulin concentration in DHM.

6.2. Materials and methods

Non–diabetic lactating mothers were recruited at Amsterdam University Medical Center (Amsterdam, The Netherlands). Written informed consent was obtained from all participants. The study protocol was approved by the local Medical Ethical Committee.

6.2.1. Sample collection and preparation

Milk samples (complete expression of all milk in one breast) were collected in disposable polypropylene bottles. If the amount was less than 200 mL, milk from multiple days was pooled. The date of milk collection was written on the bottles by the mother, and the milk was stored at −20°C. If the milk was collected at home, samples were stored at −20°C in home freezers and then transported on dry ice to the hospital. Twenty–four hours before pasteurization, the milk was thawed at 4°C. After thawing, the milk sample from each mother was divided into six tubes. One tube remained untreated as a reference. The other five tubes underwent one of the following: HoP, HTST, TUS, UV–C, or HPP. After treatment, the samples were stored at –20°C until analysis. In a preliminary study by our group, we showed that the human milk insulin concentration is not affected by freeze–thaw cycles at −20°C [6]. The processing details are described in section **S1** in the **Supplementary Appendix**.

6.2.2. Sample analysis

Macronutrient analysis was performed using a commercially available human milk analyzer (MIRIS, Uppsala, Sweden). The milk insulin concentration was determined using a luminescence immunometric assay (Atellica, Siemens Medical Solutions Diagnostics, Malvern, USA) as published previously by our group [6].

6.2.3. Statistical analysis

The milk insulin retention rate was calculated as a percentage of the insulin concentration in untreated milk, which was set at 100%. The range of agreement was set at ± 10% of the insulin concentration in untreated milk. Variables were expressed as the mean ± standard deviation (SD), median (interquartile range [IQR]), or as the frequency, depending on their distribution. The mean insulin concentrations were compared using an analysis of variance (ANOVA). A p–value <0.05 was considered to be statistically significant.

6.3. Results

Milk from 28 mothers was collected at a median of 4 (IQR, 2–7) months postpartum. The median gestational age at delivery was 39.6 (IQR, 37.5–40.9) weeks, and 10 (36%) infants were male. The median body mass index (BMI) of the mothers was 24 (20–25) kg/m^2 at the time of milk collection. The insulin retention rate was 71 ± 19% for HoP, 82 ± 20% for HTST, 101 ± 12% for TUS, 93 ± 7% for UV–C, and 106 ± 7% for HPP (**Table 1**). The mean insulin

concentration was significantly lower only in the HoP–treated samples compared to untreated milk (p=0.01).

Table 1. Effect of HoP, HTST, TUS, UV–C, and HPP on insulin in DHM.

	Untreated DHM N=28	**HoP N=28**	**HTST N=28**	**TUS N=28**	**UV–C N=28**	**HPP N=28**
Insulin concentration (pmol/L), mean ± SD	79 ± 40	53 ± 26	62 ± 33	77 ± 36	74 ± 38	84 ± 42
Retention rate (%), mean ± SD	–	71 ± 19	82 ± 20	101 ± 12	93 ± 7	106 ± 7
P–value	–	0.01	0.10	0.90	0.63	0.58

HoP, holder pasteurization; HTST, high–temperature–short–time; TUS, thermoultrasonication; UV–C, ultraviolet–C irradiation; HPP, high–pressure processing; SD, standard deviation; DHM: donor human milk

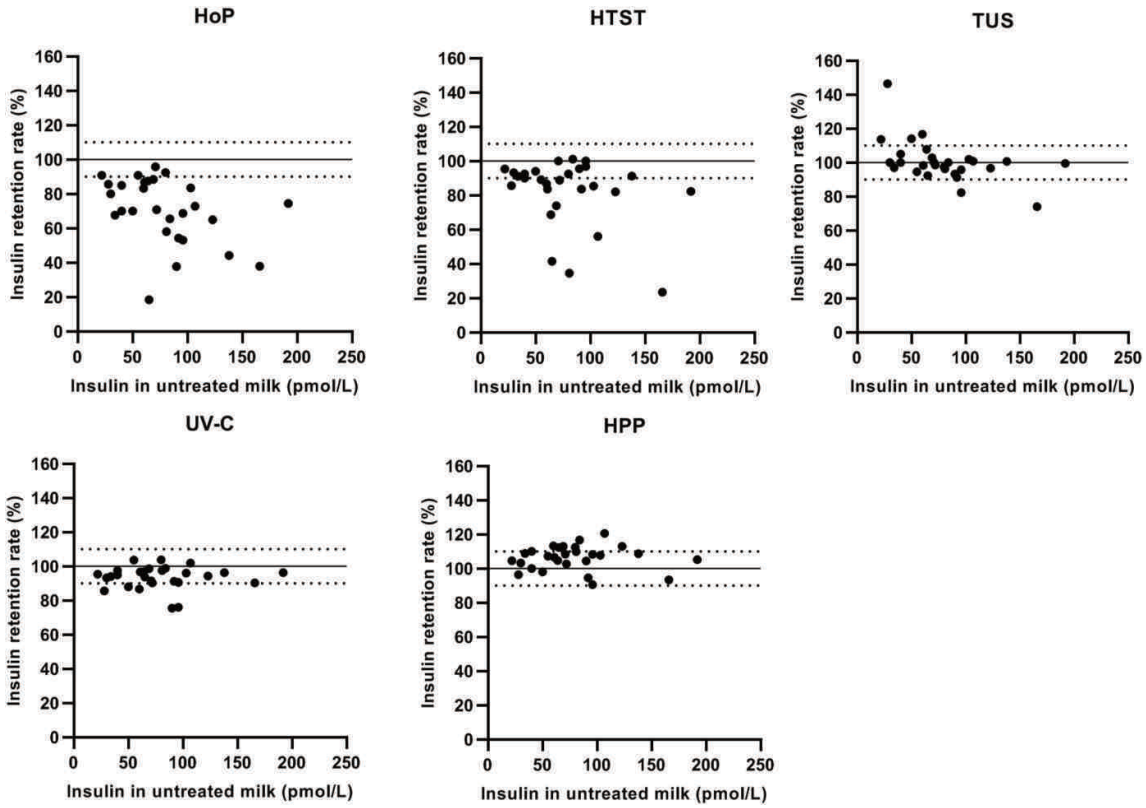

Figure 1. The human milk insulin retention rate after HoP (A), HTST (B), TUS (C), UV–C (D), and HPP (E) (n=28). The insulin retention rate was calculated as a percentage of the insulin concentration in untreated milk which was set at 100% (identity line). The range of agreement was set at ±10% of the insulin concentration in untreated milk (dashed lines). HoP, holder pasteurization; HTST, high–temperature–short–time; TUS, thermoultrasonication; UV–C, ultraviolet–C irradiation; HPP, high–pressure processing; SD, standard deviation

Figure 1 shows the retention rate of all individual milk samples after either HoP, HTST, TUS, UV–C, and HPP. Samples with a retention rate that was within the range of agreement were as follows: 4 (14%) for HoP, 13 (46%) for HTST, 22 (79%) for TUS, 23 (82%) for UV–C, and 21 (75%) for HPP. A decreased milk insulin concentration (i.e., retention rate <90%) was observed in 24 (86%) of the samples treated with HoP, 15 (54%) of the samples treated with HTST, 2 (7%) of the samples treated with TUS, and 5 (18%) of the samples treated with UV–C. None of the HPP–treated samples showed a decreased insulin concentration. An increased milk insulin concentration (i.e., retention rate >110%) was observed in 4 (14%) TUS–treated samples and 7 (25%) HPP–treated samples.

6.4. Discussion

To the best of our knowledge, this is the first study that investigated the effect of HoP, HTST, TUS, UV–C, and HPP on the insulin concentration in DHM simultaneously. TUS, UV–C, and HPP did not affect the DHM insulin concentration, while the insulin concentration was decreased by HoP and HTST. The insulin concentration in DHM was affected to a larger extent by HoP than by HTST.

All techniques must meet the highest safety standards because vulnerable preterm infants are the usual recipients of DHM. In our preliminary study, the application of TUS (60 W; 6 min), UV–C (4683 J/L), and HPP (500 MPa; 5 min), resulted in a 5–log10 reduction of *Enterobacteriaceae* and *Staphylococci* species as required by human milk banking guidelines (**Table S1** in the **Supplementary Appendix**). The HTST parameters that are used showed similar results as HoP for eliminating microbial contaminants in human milk in a previous study [3].

The milk insulin concentration was significantly decreased after HoP, with an average retention rate of 71%. This is consistent with two previous studies, which showed a retention rate of 68% and 54% respectively after HoP [2]. The mean milk insulin concentration after HTST treatment was not significantly different from untreated milk, but the mean retention rate was outside the range of agreement, which suggests milk insulin degradation. The insulin degradation during HoP and HTST is probably caused by the thermal instability of insulin at a temperature above 60°C [7]. The temperature during TUS remained below this insulin thermal instability threshold, and no heating of the samples occurred during either HPP or UV–C, which may explain the milk insulin preservation when applying these innovative methods [7].

A small milk insulin concentration increase was observed in 4 (14%) and 7 (25%) samples treated with TUS and HPP respectively. This is comparable to the milk leptin concentration increase after HPP treatment as observed by Wesolowska et al. [8]. They hypothesized that the increase might be caused by a small release of leptin incorporated in fat globules due to the high pressure. A similar process might occur with other hormones, such as insulin. However, additional studies are needed to investigate this hypothesis.

The biological effect of milk insulin has been investigated *in vitro* and in small–scale *in vivo* experiments [4]. The small intestinal mass and intestinal disaccharidase activity were significantly higher in piglets and rats that were treated with either enteral recombinant human insulin (rh–insulin) or recombinant porcine insulin compared to the respective control [4]. The effect on the intestine seems to be mediated by insulin receptors, which has been observed on either the apical or basolateral membrane of enterocytes [4]. Consistent with animal studies, the

intestinal lactase activity was significantly higher in six preterm infants who received enteral rh–insulin compared to a historical control group [4]. In addition, time to achieve full enteral feeding was significantly reduced in preterm infants who received rh–insulin–supplemented formula compared to placebo–supplemented formula in a small clinical trial [5]. Thus, milk insulin has been suggested to be a key factor for optimal intestinal development and function, especially in preterm infants. Therefore, the higher occurrence of feeding intolerance and growth restriction in preterm infants fed DHM relative to preterm infants fed their mother's own milk might be because DHM is generally treated by HoP, which significantly decreases the insulin concentration [9].

Besides insulin, several other bioactive factors in DHM were shown to be better preserved by innovative techniques compared to HoP. For example, UV–C (4863 J/L) did not affect bile salt–stimulated lipase (BSSL) or alkaline phosphatase activity. Additionally, the concentrations of secretory immunoglobulin (Ig) A, lactoferrin, and lysozyme levels in DHM were higher after UV–C compared to HoP [10]. Furthermore, the IgA, IgG, and IgM retention rates were significantly higher in DHM after HPP treatment (500 MPa; 5 min) compared to HoP [11]. Additional studies are needed to investigate whether the improved DHM quality results in improved clinical outcomes of preterm infants who are fed DHM.

In conclusion, TUS, UV–C, and HPP all completely preserve milk insulin in DHM. The insulin concentration in DHM is affected to a larger extent by HoP than by HTST. These results indicate that TUS, UV–C, and HPP may be alternatives to HoP. Additional studies are needed to further investigate the effect of these methods on DHM safety and on the preservation of other DHM bioactive components before implementing them in human milk banks.

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Conflict of interest

J.B.v.G. is member of the National Health Council, Chair of the Committee on Nutrition and Pregnancy, and Director of the Dutch National Human Milk Bank. He does not receive any honorarium for his services.

The supplementary data can be found online at:
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Chapter 7

Human milk from previously COVID–19–infected mothers: The effect of pasteurization on specific antibodies and neutralization capacity

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Abstract

Since the outbreak of coronavirus disease 2019 (COVID–19), many put their hopes in the rapid availability of effective immunizations. Human milk, containing antibodies against syndrome coronavirus 2 (SARS–CoV–2), may serve as means of protection through passive immunization. We aimed to determine the presence and pseudovirus neutralization capacity of SARS–CoV–2 specific IgA in human milk of mothers who recovered from COVID–19, and the effect of pasteurization on these antibodies.

This prospective case control study included lactating mothers, recovered from (suspected) COVID–19 and healthy controls. Human milk and serum samples were collected. To assess the presence of SARS–CoV–2 antibodies we used multiple complementary assays, namely ELISA with the SARS–CoV–2 spike protein (specific for IgA and IgG), receptor binding domain (RBD) and nucleocapsid (N) protein for IgG in serum, and bridging ELISA with the SARS–CoV–2 RBD and N protein for specific Ig (IgG, IgM and IgA in human milk and serum). To assess the effect of pasteurization, human milk was exposed to Holder (HoP) and High–Pressure Pasteurization (HPP).

Human milk contained abundant SARS–CoV–2 antibodies in 83% of the proven cases and in 67% of the suspected cases. Unpasteurized milk with and without these antibodies was found to be capable of neutralizing a pseudovirus of SARS–CoV–2 in (97% and 85% of the samples respectively). After pasteurization, total IgA antibody levels were affected by HoP, while SARS–CoV–2 specific antibody levels were affected by HPP. Pseudovirus neutralizing capacity of the human milk samples was only retained with the HPP approach. No correlation was observed between milk antibody levels and neutralization capacity.

Human milk from recovered COVID–19–infected mothers contains SARS–CoV–2 specific antibodies which maintained neutralization capacity after HPP. All together this may represent a safe and effective immunization strategy after HPP.

Keywords: immunoglobulins; pasteurization; COVID–19; breastfeeding

7.1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS–CoV–2) outbreak, which was first reported in December 2019, has had an enormous global impact. SARS–CoV–2 can cause coronavirus disease 2019 (COVID–19) with the number of confirmed cases over 130 million, and over 2.8 million deaths globally as of April 2021. In response to the pandemic, many countries have had to introduce drastic lockdowns to enforce physical separation, affecting economies worldwide, while also imposing a huge psychological burden on specific groups such as the elderly and school–aged children. On a personal level, general preventive measures like protective materials, physical distancing and frequent hand washing, have shown to be effective. As these measures are not sustain– able for prolonged periods of time, the pandemic has necessitated rapid development of effective vaccines as prevention. Even with the development of several COVID–19 vaccines and extensive vaccination efforts, we are still far from global vaccination goals [1].

When looking at preventive strategies, it is interesting to note that, in infants, breast–feeding is associated with a 30% reduction in respiratory infections when compared to formula feeding [2,3]. It is generally accepted that this protective effect is due to the hu– man immune components in human milk, such as specific antibodies of which secretory immunoglobulin A (sIgA) is the most abundant. SIgA represents our first line of defense as it acts directly at mucosal surfaces [4]. SIgA inhibits microbial binding to host receptors of intestinal epithelial cells, entrapping pathogenic microorganisms within the mucus and enhancing ciliary activities, thus eliminating invading pathogens [5–7]. Through this mechanism, human milk sIgA may provide protection against entry of SARS–CoV–2 in the airway at mucosal surfaces.

The structural proteins of SARS–CoV–2 include the spike (S), nucleocapsid (N), membrane, and envelope proteins (**Figure 1**). The S1 subunit of the S protein contains the receptor binding domain (RBD), which facilitates angiotensin–converting enzyme 2 (ACE2) receptor mediated virus attachment, while the S2 subunit of the S protein promotes mem– brane fusion to initiate the infection of host cells. The N protein encapsulates viral RNA and is necessary for viral transcription and replication [8]. The human immune system will, when infected by SARS–CoV–2, generate antibodies against one or more of these viral proteins, whereby variability may exist in the immunoglobulin class preferentially made (e.g., IgG, IgA, IgM) and the antigen to which the immunoglobulin binds. The titers of antibodies are individual specific, but also amongst others determined by the severity of the infection and the time that has passed since the onset of the infection. Therefore, it is recommended to use complementary assays to determine SARS–CoV–2 antibody titers.

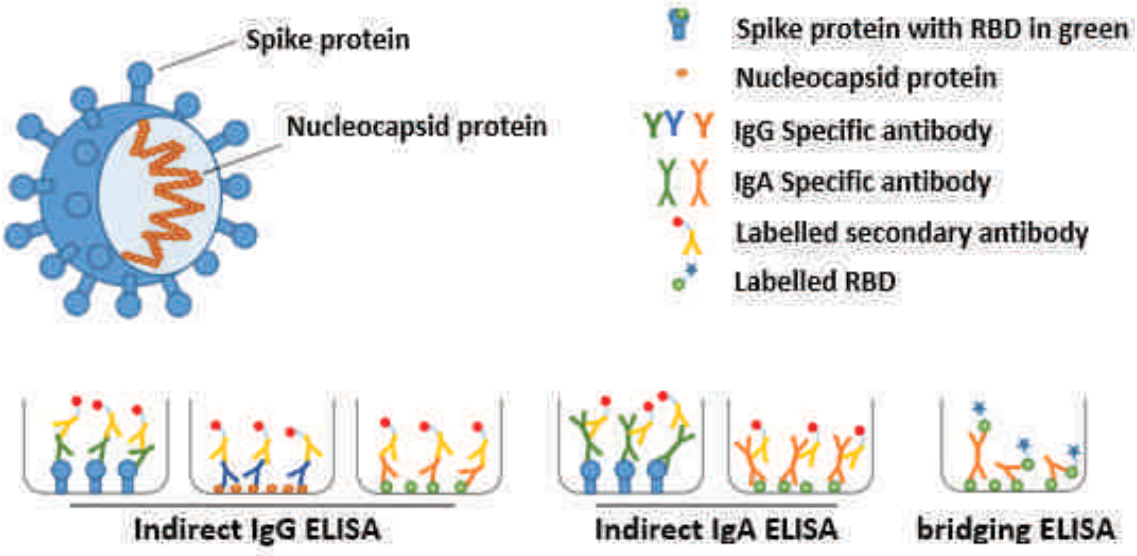

Figure 1. Schematic representation of SARS–CoV–2 and the different ELISA assays used to detect SARS–CoV–2–reactive antibodies. The spike (including the receptor binding domain (RBD)) and nucleocapsid proteins of SARS–CoV–2 are depicted in the context of the virus. SARS–CoV–2 specific antibodies were detected using multiple complementary ELISA assays. The indirect ELISA assays using S, RBD or N were used to detect IgG or IgA specific–antibodies (green, blue or orange, respectively) and the bridging ELISA assay was used to detect total Ig against the RBD

There is strong evidence that antibodies, especially of the IgA class, against several respiratory infections, such as influenza, are secreted into human milk [9,10]. A previous study

indicated that 15–30 days following the onset of symptoms, antibodies against the SARS–CoV–2 RBD may be present in human milk [11]. These data form the basis of our hypothesis that an array of SARS–CoV–2–reactive antibodies may be present in human milk from mothers who have recovered from COVID–19. Human milk sIgA may provide insights into clinical strategies to reduce the incidence of SARS–CoV–2 infections, by neutralizing the virus in the airway mucosa, although many steps need to be taken before such an approach can be implemented. This is an intriguing perspective as monoclonal antibody therapies can provide a means of treatment for the disease even after a vaccine is available, particularly in vulnerable populations. However, human milk may contain pathogens, and therefore pasteurization is required prior to use [12]. Holder pasteurization (HoP), a heat treatment at 62.5 ºC for 30 min, is currently the standard pasteurization method for human milk [13]. Although HoP effectively inactivates microbial contaminants, it concomitantly reduces the activity of some important bioactive milk components [13,14]. To prevent such reduced activity, alternative methods to HoP, such as high–pressure pasteurization (HPP), are currently being investigated [15,16]. Our aim was thus to evaluate the level of SARS–CoV–2 reactive antibodies and determine efficacy of virus neutralization in serum, unpasteurized human milk, and in human milk after thermal (HoP) and non– thermal (HPP) pasteurization.

7.2. Materials and Methods

7.2.1. Study Population

This prospective case control study aimed to include 40 lactating women with a confirmed or high probability of COVID–19. Lactating women who recovered from a proven COVID–19 infection were recruited by an online recruitment letter. A confirmed infection was defined as a positive SARS–CoV–2 PCR from a nasal–pharyngeal swab. Subjects were classified in the suspected COVID–19 group in the event of a confirmed infection with SARS–CoV–2 in the household and if the lactating woman developed COVID–19 symptoms. A control group, of 15 healthy lactating women, was recruited simultaneously as the proven COVID–19 infected group from the Amsterdam UMC if they met the following criteria: lactating women who delivered at Amsterdam UMC with a negative SARS–CoV–2 PCR from a nasal–pharyngeal swab during delivery, and without symptoms of COVID–19. Ethical approval was obtained from the Medical Ethics Committee of the Amsterdam UMC, location VUmc and written informed consent was obtained from all participants.

7.2.2. Material Collection

All participants were requested to collect 100 mL of human milk in specially provided bottles and to store the bottle in their freezer until collected by study staff during a home visit. Subsequently, the samples were stored at −20 ºC. During the home visit, maternal serum was collected by a trained phlebotomist.

7.2.3. Laboratory Analyses

2.3.1. Evaluation of Antibodies in the Serum and Human Milk

To assess the diversity and variability of antibodies present in human milk and serum we decided to use multiple complementary assays. First, ELISA with the SARS–CoV–2 spike

protein to detect specific IgA and IgG in human milk and serum, respectively. Second, ELISA with the SARS–CoV–2 receptor binding domain (RBD) and with the SARS–CoV–2 nucleocapsid (N) protein for specific IgG (serum only). Third, a bridging ELISA with the SARS–CoV–2 RBD and N protein for specific total Ig (IgG, IgM and IgA in human milk and serum). All of the ELISA based assays used are depicted in **Figure 1** and described in more detail below.

7.2.3.2. Detection of Anti–SARS–CoV–2 Ig in Serum and Human Milk with ELISA

Soluble prefusion–stabilized S–protein of SARS–CoV–2 were generated as previously described [17]. This protein was immobilized on a 96–well plate (Greiner, Kremsmünster, Austria) at 5 µg/mL in 0.1 MNaHCO3 overnight, followed by a one–hour blocking step with 1% casein PBS (Thermo Scientific, Waltham, MA, USA). Human milk was diluted 1:5 and serum was diluted 1:100 in 1% casein PBS and incubated on the S–protein coated plates for 2 h to allow binding. Antibody binding was measured using 1:3000 diluted HRP– labelled goat anti–human IgG (Jackson Immunoresearch, West Grove, PA, USA) in casein for the serum samples and 1:3000 diluted HRP–labelled goat anti–human IgA (Biolegend, San Diego, CA, USA) in casein for the human milk samples. The healthy controls (serum and human milk) were used to determine cut–off values defined as the mean plus two times the standard deviation. Specificity of the ELISA was shown to be >95% for both serum and human milk and the sensitivity was >90% for serum and >80% for human milk.

7.2.3.3. Bridging ELISA with the SARS–CoV–2 RBD and Nucleocapsid Protein

Antibodies against RBD protein were measured as total Ig (IgG, IgA and IgM) ELISA and an IgG ELISA as described previously [18]. Briefly, for total antibodies, samples were incubated (1:10 for serum, undiluted for human milk) on plates coated with RBD protein (produced in–house [18]) and specific antibodies were subsequently detected using biotinylated RBD protein (produced in–house [18]). For the IgG ELISA, serum samples were diluted 1:100 and incubated on RBD–coated plated, followed by detection of specific IgG antibodies using a mouse monoclonal anti–human IgG antibody (produced in–house [18]). Total Ig against N protein in serum was measured in 1:10 diluted serum on N protein– coated plates followed by detection using biotinylated N protein. In order to determine the cut–off values, pre–pandemic controls were used to provide ~99% specificity as previously described [18]. The results of the pre–pandemic controls are not described in this paper.

7.2.3.4. Effect of Antibodies on Virus Replication

Pseudovirus Neutralization Assay

Neutralization assays and the generation of a SARS–CoV–2 pseudovirus containing a NanoLuc luciferase reporter gene were performed as previously described [19]. Briefly, HEK 293T cells (ATCC, CRL–11268) were transfected with a pHIV–1NL43ΔENVNanoLuc reporter virus plasmid and a SARS–CoV–2–SΔ19 plasmid. Cell supernatant containing The pseudovirus was harvested 48 h post transfection, centrifuged for 5 min at 500× g and sterile filtered through a 0.22 µm pore size PVDF syringe filter. For neutralization assays HEK 293T expressing the SARS–CoV–2 receptor ACE2 (HEK 293T/ACE2 [19]) were cultured in DMEM (Gibco), supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and

streptomycin (100 µg/mL).To determine the neutralization activity in serum or human milk, HEK 293T/ACE2 cells were first seeded in 96–well plates coated with 50 µg/mL poly–l–lysine at a density of 2×104/well in the culture medium as described above, but with GlutaMax (Gibco) added. The next day, duplicate serial dilutions of heat inactivated serum or human milk samples were prepared in the same medium as used for seeding of cells and mixed 1:1 with ∼1 × 103 infectious units pseudovirus. This mixture was incubated at 37 ºC for 1 h before adding it to the HEK 293T/ACE2 cells in a 1:1 ratio with the cell culture medium. After 48 h, the cells were lysed and luciferase activity was measured in the lysates using the Nano–Glo Luciferase Assay System (Promega, Madison, WI, USA). Relative luminescence units (RLU) were normalized to those from cells infected with SARS–CoV–2 pseudovirus in the absence of sera/saliva/swabs. Neutralization titers (ID50–values) were determined as the serum dilution at which infectivity was inhibited by 50%.

Replication Inhibition of a SARS–CoV–2 Clinical Isolate Assay

In order to assess if human milk with SARS–CoV–2 specific antibodies possess virus neutralizing activity, in vitro neutralizing assays were conducted using a SARS–CoV–2 clinical isolate strain, which was kindly provided by Christian Drosten, Charité–Universitätsmedizin, Berlin, Germany (BetaCoV/Munich/BavPat1/2020), performed under biosafety level 3+ conditions. In brief, 60 µL of SARS–CoV–2 working dilution containing approximately 200 TCID50/well was mixed with 60 µL of serially 2–fold dilutions of heat–inactivated serum or milk, in triplicates and incubated for 60 min at 37 ºC to allow for neutralization of the virus. Subsequently 100 µL of these virus/antibody mixtures were added to confluent VERO E6 cell monolayers (ATCC; CRL–1586) and incubated at 37 ºC for four to six days. The virus working dilution and the original virus stock were titrated in a parallel plate and served as positive virus controls in each assay run. After incubation at 37 ºC 20 µL of a WST–8 Cell Counting Kit–8 (CCK–8) solution (Sigma–Aldrich, St. Louis, MO, USA; 96992) was added to each well of the plate, followed by an incubation for three hours at room temperature. The absorbance at 450 nm was measured using microplate reader (Synergy H1, Biotek, Winooski, VT, USA). The Reed and Muench method was used to determine the 50% endpoint titer of the sample, as well as the virus titer (stock and back titration).

7.2.3.5. Evaluation of the Effect of Pasteurization of Human Milk on SARS–CoV–2 Antibodies

To assess the effect of pasteurization we used two methods of pasteurization on all of the collected human milk samples, after treatment we evaluated the amount of SARS–CoV–2 antibodies and neutralizing capacity of human milk between raw milk and pasteurized milk samples. During Holder pasteurization (HoP), using current standard methods, human milk is pasteurized at 62.5 ºC for 30 min. An alternative to HoP pasteurization is high pressure pasteurization (HPP), which inactivates vegetative (including pathogenic) micro–organisms, yeasts, molds and viruses, without causing heat–induced damage [20].

Samples were stored frozen at −20 ºC and thawed overnight in a refrigerator (7 ºC), prior to being transferred into sterile pouches that were double packed and treated in a pilot–scale high–pressure unit with water at ambient temperature [21]. We applied a hydrostatic pressure of 500 MPa for 5 min. All samples were stored at −20 ºC directly after treatment.

7.2.3.6. Monitoring IgA Clone Diversity in Human Milk by Mass Spectrometry

In addition to the classical antibody detection assays, we used a novel mass spectrometry (MS) method to examine the IgA clonal diversity in human milk. We examined the IgA clones in unpasteurized human milk, and after the two different pasteurization techniques.

The antigen binding fragments (Fab) were proteolytically released from the captured IgAs, and the resulting Fab fragments (45–50 kDa) of individual clones were profiled using MS. The abundance of each unique detected clone could be determined, and thus for each clone the effect of the two different pasteurization techniques could be monitored.

Stabilized spike protein from SARS–CoV–2 was produced and purified as described before [22]. The Spike protein was coupled to NHS–activated agarose for two hours at room temperature. Free NHS groups were inactivated by incubation with 1 M Tris for 30 min. Spike recognizing molecules were captured from milk by incubating 250 µL unpasteurized milk and 100 µL PBS for 2 h end–over–end. The non–binding fraction was collected by centrifugation, and the SARS–CoV–2 specific antibodies were eluted using 100 mM Glycine– HCl (pH 2.7) and immediately neutralized with 1 M Tris (pH 8.0). Next, the IgA antibodies were captured on CaptureSelect IgA affinity matrix from the neutralized eluate (substituted with blocking powder, final concentration 1%), from 140 µL of the non–binding fraction, or from 100 µL unpasteurized milk. Then, Fab portions were generated as described above, collected by centrifugation, and immediately measured on the mass spectrometer.

7.2.4. Statistical Analysis

Patient characteristics and COVID–19 symptoms were expressed as mean with standard deviation (SD) or median with interquartile range (IQR) depending on their distribution. Statistical analysis was performed with IBM SPSS Statistics for Windows, version 26 (IBM Corp., Amonk, NY, USA). In order to compare SARS–CoV–2 IgA in unpasteurized milk of the cases and controls, a Mann–Whitney U test was performed in GraphPad Prism 8.2.1. In order to evaluate the effect of pasteurization on antibody level and neutralization capacity, statistical analyses were performed, depending on the distribution of the data. A Wilcoxon matched–pairs signed rank test was performed in order to compare IgA retention according to LC/MS profiles following HoP and HPP and to compare Spike IgA titers between HPP and HoP milk as a % relative to UP. These tests were performed in Python 3.8.8, Pandas 1.2.3, Numpy 1.19.2, Scipy 1.6.1, visualized with Matplotlib 3.3.4 and Seaborn 0.11.1. Differences in neutralization capacity of the pseudovirus of human milk after pasteurization was tested with a Friedman test in SPSS Statistics for Windows, version 26. To test which groups differ, a Dunn's post hoc test was performed. Differences in neutralization capacity of the pseudovirus between the confirmed cases, suspected cases and controls was tested with a Kruskal Wallis test in SPSS Statistics for Windows.

7.3. Results

Our prospective case control study included 40 lactating women with confirmed or a high probability of COVID–19 and 15 healthy controls during pandemic. Four women had active COVID–19 symptoms on the day of the scheduled house visit, therefore we were not able to collect the body materials of these women, resulting in a final study population of 38 cases and

13 controls. Three of the subjects with a confirmed infection were admitted to the hospital. For all subjects, samples were obtained at different time intervals from the onset of clinical symptoms, as shown in **Table 1**.

7.3.1. SARS–CoV–2 Antibodies in Human Milk and Serum

Human milk contained antibodies against the SARS–CoV–2 virus, using any of the assays, in 24 out of 29 (83%) proven cases and in six out of nine (67%) of the suspected cases (**Figure 2**). A large variability in antibody levels was found in the milk samples of all subjects. Both the assay assessing IgA response against the S protein and the assay detecting the total Ig response against RBD showed a variable pattern in antibody type and the SARS–CoV–2 protein it recognized (**Supplemental Table S1**). The median and range of SARS–CoV–2 Spike–IgA and RBD–Ab in unpasteurized milk from the case and control groups ($p < 0.001$) are depicted in **Figure 3**.

Table 1. Patient characteristics of the lactating women with a confirmed or highly probable COVID–19 and controls.

Characteristics	**Confirmed COVID–19** ***N = 29***		**Suspected COVID–19** ***N = 9***		**Controls** ***N = 13***
Gestational age—weeks media (IQR)	39.7 (38.5, 40.7)		38.8 (36.8, 40.4)		40.7 (39.6, 41.1)
Age of child—weeks median (IQR)	28.9 (12.1, 39.5)		12.6 (8.4, 40.7)		6.1 (4.3, 7.4)
Age of mother—years mean (SD)	31.1 (3.1)		30.3 (4.1)		33.2 (3.3)
Time between start of clinical symptoms and collection of human milk—weeks mean (SD)	5.9 (2.6)		5.7 (2.1)		NA
Symptoms and duration in days	*N*. (%)	Median (IQR)	*N*. (%)	Median (IQR)	
Fever >37.5 °C	21 (72%)	3 (1, 5)	6 (67%)	1 (0, 4)	NA
Cold	24 (83%)	12 (5, 20)	4 (44%)	7 (4, 54)	NA
Cough	21 (72%)	14 (5, 28)	5 (56%)	6 (3, 12)	NA
Sore throat	21 (72%)	6 (4, 14)	5 (56%)	6 (3, 12)	NA
Tachypnea	5 (17%)	11 (4, 14)	2 (22%)	NA	NA
Dyspnea	14 (38%)	7 (3, 28)	1 (11%)	NA	NA
Stomachache	5 (17%)	2 (1, 9)	2 (22%)	NA	NA
Nausea	5 (17%)	3 (2.5, 14)	1 (11%)	NA	NA
Vomiting	2 (7%)	NA	0	NA	NA
Diarrhea	5 (17%)	2 (1, 25)	3 (33%)	NA	NA
Headache	24 (83%)	5 (2, 12)	8 (89%)	7 (4, 14)	NA
Photophobia	2 (7%)	NA	0	NA	NA
Anosmia	18 (62%)	20 (13)	6 (67%)	9 (4, 22)	NA
Ageusia	17 (59%)	19 (13)	4 (44%)	14 (11, 25)	NA
Fatigue	24 (83%)	20 (16)	8 (89%)	10 (4, 36)	NA
Anorexia	10 (34%)	12 (8, 21)	3 (33%)	NA	NA
Hospital admission	3 (10%)	NA	0	NA	NA
Non–invasive respiratory support (O2) during admission	2 (7%)	NA	0	NA	NA

With a complementary method, using the spike protein from SARS–CoV–2 to enrich for antibodies from milk, analyzing their Fab fragments subsequently by LC–MS, we were able to demonstrate the presence of a few high abundant SARS–CoV–2 antigen–specific antibodies in milk of COVID–19 recovered women (**Figure 4**).

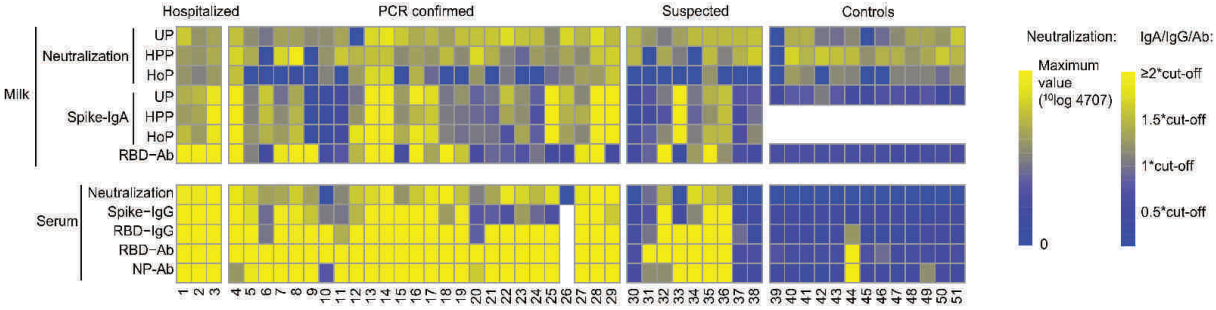

Figure 2. Multiple assay assessment of SARS–CoV–2 antibody levels in human milk and serum. Colors from blue to yellow indicate increasing levels of antibodies in milk and serum, relative to the cut–off values of the respective assays, with all levels over 2 times the cut–off being bright yellow, as indicated by the color scale. For the neutralization of the pseudovirus by milk (unpasteurized (UP), high pressure pasteurized (HPP) and holder pasteurized (HoP)) or serum, colors from blue to yellow indicate increasing neutralization capacity. Ab; total Ig. * multiplication sign.

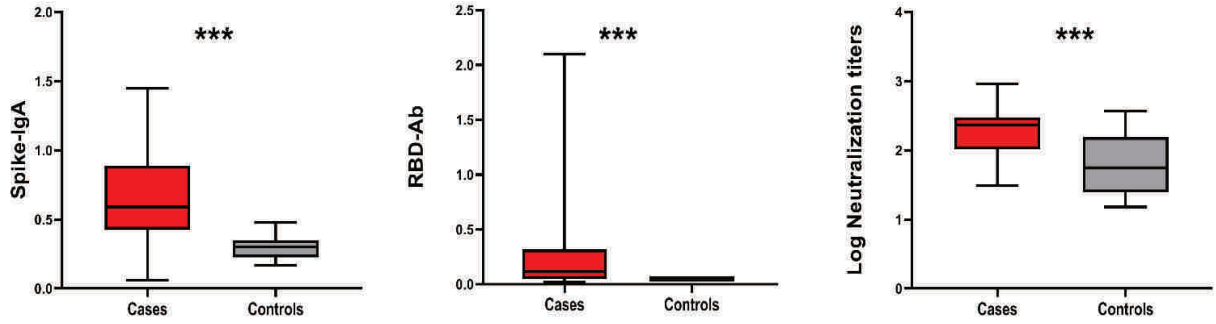

Figure 3. Assessment of human milk antibody interaction with SARS–CoV–2 proteins in unpasteurized milk from the case and control groups. All box plots depict the interquartile range (IQR) as a median value with a lower 25th and upper 75th quartile range, and lower and upper whiskers to indicate the variability outside of the IQR. All cases are depicted in red and all controls are depicted in grey. *** indicates a p–value < 0.001 (a) OD450 nm SARS–CoV–2 Spike–IgA ($p < 0.001$), (b) OD450 nm SARS–CoV–2 RBD–Ab ($p < 0.001$) and (c) neutralization titers in unpasteurized milk of the cases and controls ($p < 0.001$).

Antibody secretion may well be dependent on the time that has passed since the onset of COVID–19 and the severity. By using a cross sectional sampling design, we were able to show that even up to 13 weeks from disease onset, detectable levels of antibodies were found in both human milk and serum (**Figure 5**). While over 80% of the human milk samples in the PCR proven cases contained antibodies, all of their blood samples showed a positive response in at least one of the assays.

7.3.2. Pasteurization of Human Milk and IgA Antibodies

Following the detection of antibodies in milk, we aimed to quantify the effect of different pasteurization methods on the antibody levels. Using mass spectrometry techniques we noticed

that following HoP and HPP, the relative abundance of each clone remained largely unaffected, although the actual concentration of most clones, and thus of total IgA, was slightly lowered for both methods of pasteurization relative to UP milk samples (**Figure 6a**). The reduction in overall IgA concentration was greater in HoP– than HPP–treated milk. In contrast, we did observe a significant difference in the levels of IgA anti S protein antibodies after both methods of pasteurization, with lower levels in HPP milk compared to HoP milk (**Figure 6b** and **Supplemental Figure S1**).

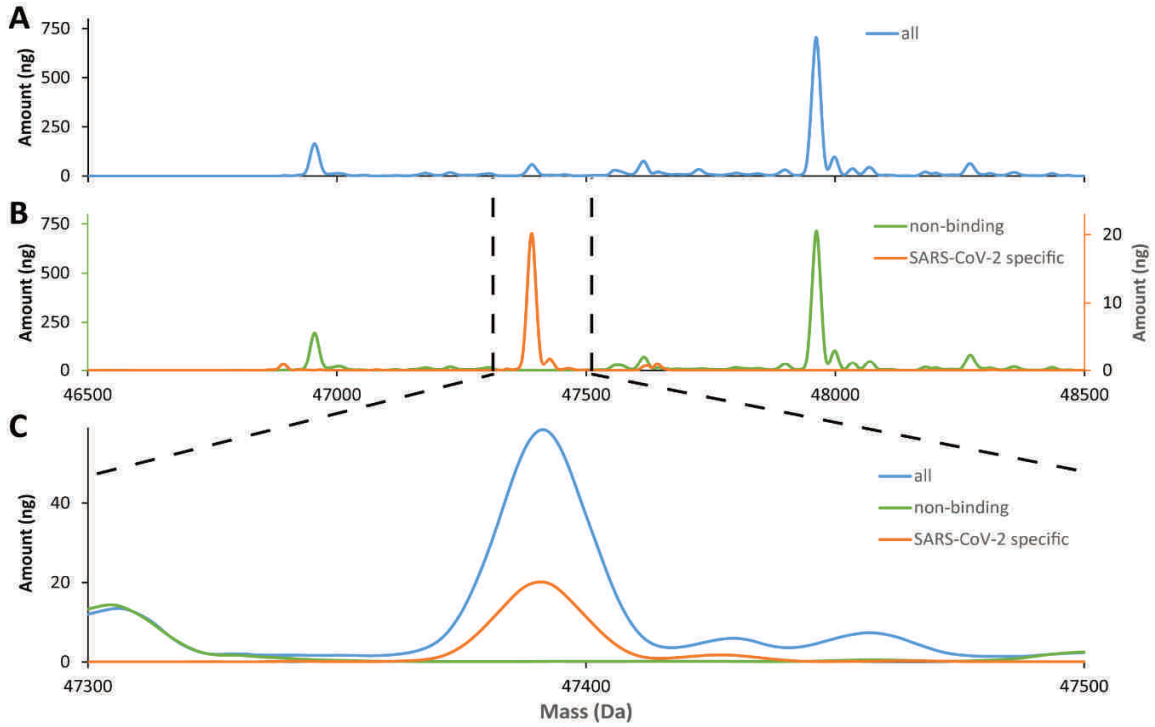

Figure 4. LC–MS profiles of Fab fragments originating from IgA clones present in human milk. (A) Profile of IgA clones detected in the unpasteurized milk of Patient 1; (B) SARS–CoV–2 antigen–specific affinity purification yields specific clones (orange line; right y–axis), distinct from the depleted flow–through fraction (green line, left y–axis); (C) Overlay of the three profiles reveals the high specificity of a few antigen–specific clones.

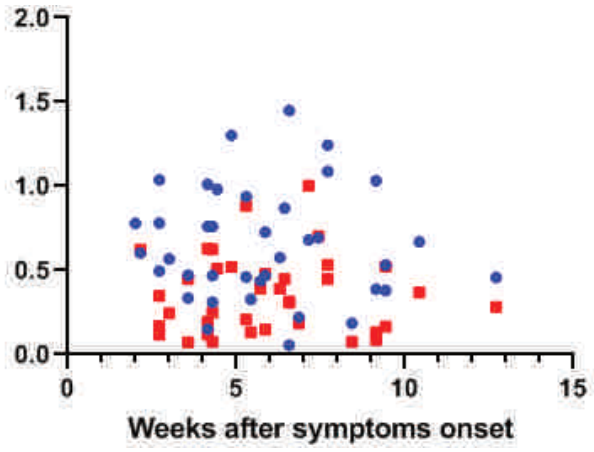

Figure 5. Detection of antibody levels in human milk and serum relative to onset of COVID–19 symptoms. The OD450 nm values for human milk spike protein IgA (blue dots) and serum IgG (red squares) levels from ELISA are plotted against the sampling time point in weeks after the onset of COVID–19 symptom.

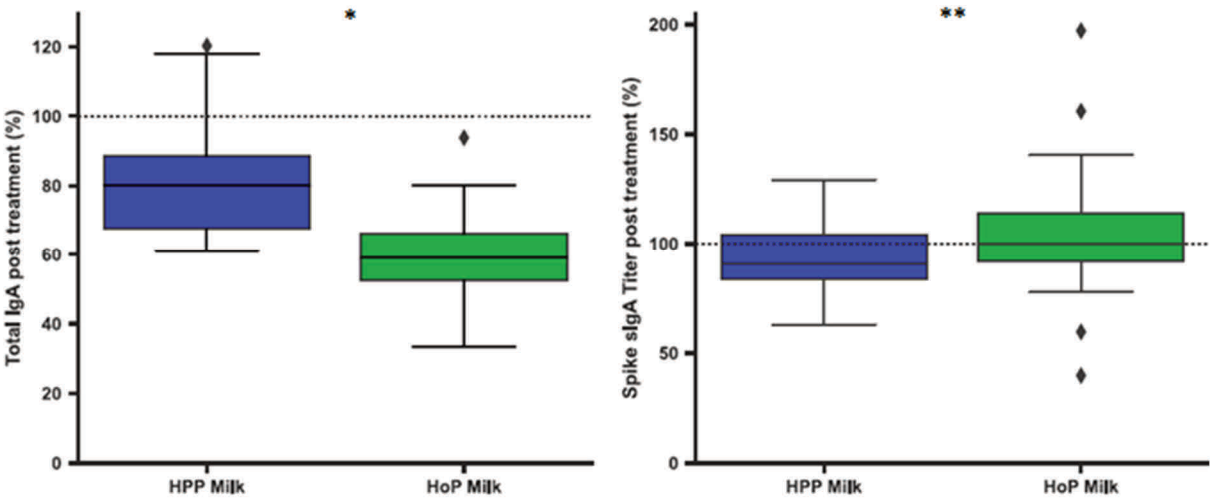

Figure 6. Assessment the effects of pasteurization on human milk IgA levels. All figures are shown as box and whisker plots as median and IQR for human milk expressed as the percentage of treated relative to untreated human milk. ♦ are values outside the IQR. The * indicates a p–value < 0.05, the ** a p–value < 0.01. (a) IgA retention according to LC–MS profiles following HoP (n = 9) and HPP (n = 9) (p = 0.020) (b) Spike IgA titers following HoP (n = 38) and HPP (n = 38) (p = 0.006)

7.3.3. SARS–CoV–2 Virus Neutralization in Unpasteurized and Pasteurized Milk

Next, we aimed to assess if human milk that contained antibodies against SARS–CoV–2 was able to reduce virus replication using two different models. First, neutralization of a SARS–CoV–2 pseudovirus was determined for both serum and human milk (**Figures 2** and **7**). In both cases (proven and suspected) and controls, neutralization capacity was observed in unpasteurized milk, although, the median of the ln transformed neutralizing capacity, defined as 50% inhibitory dilution, was comparable in milk from women with proven COVID–19 2.36 (IQR 2.03–2.53) and milk from women with a suspected infection 2.37 (IQR 1.96–2.39) and higher compared with controls 1.75 (IQR 1.39–2.19) ($p = 0.009$). Neutralization capacity differed between unpasteurized and pasteurized milk ($p < 0.0001$) and was generally better preserved after the HPP ($p = 0.906$). In contrast, after HoP a substantial decrease in neutralization capacity was observed ($p < 0.001$).

Second, using a replication inhibition with a SARS–CoV–2 clinical isolate, the inhibitory capacity of human milk was determined (**Supplemental Figure S2**). In seven out of the 38 (18%) unpasteurized milk samples, and in eight out of the 38 (21%) HPP milk samples, neutralization capacity was observed. None of the HoP milk samples showed neutralization capacity. Some (n = 3) HPP milk samples did show neutralizing capacity while the same sample before pasteurization did not exert such an effect. We could not detect a linear correlation between milk antibody levels and virus neutralizing capacity.

All these data together indicate that heat treatment may have caused a loss–of–function even though this did not result in reduced antibody levels according to the preformed ELISA assays. This shows the importance of testing the functionality of the milk in a neutralization assay in addition to the analysis of the antibody levels.

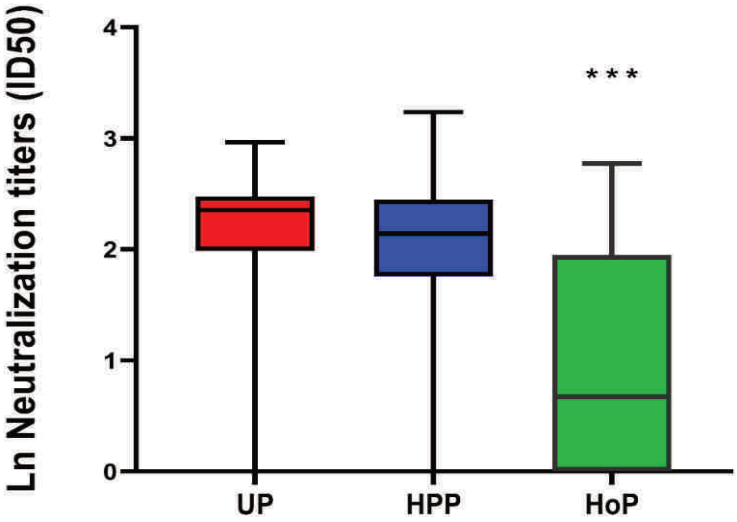

Figure 7. SARS–CoV–2 virus neutralization in unpasteurized and pasteurized human milk. The median and interquartile ranges of neutralizing activity against the pseudovirus, expressed as ln neutralizing capacity (50% inhibitory dilution), for unpasteurized (UP) milk, high pressure pasteurized (HPP) milk and Holder pasteurized (HoP) milk of PCR positive and suspected participants (n = 38). Neutralization capacity was generally preserved after the HPP pasteurization ($p = 0.906$) and not after HoP ($p < 0.001$) ***, relative to UP milk

7.4. Discussion

We demonstrate that human milk of mothers who recovered from COVID–19 contains significant amounts of IgA against SARS–CoV–2, for at least 13 weeks following the onset of COVID–19 symptoms. After pasteurization, total IgA antibody levels were affected by HoP, while SARS–CoV–2 specific antibody levels were affected by HPP. Pseudovirus neutralizing capacity of the human milk samples was only retained with the HPP approach. No correlation was observed between human milk antibody levels and neutralization capacity.

We also show that human milk samples from some donors had neutralizing capacity against a SARS–CoV–2 clinical isolate in the stringent replication inhibition as–say, resulting in significant inhibition of virus propagation, while most donors had human milk and serum neutralizing capacity against a SARS–CoV–2 pseudovirus. Interestingly, neutralizing capacity was also observed in human milk samples of the control group, which can be explained by the presence of other antiviral proteins, be–sides antibodies [23]. Only few human milk samples with SARS–CoV–2 antibodies were able to neutralize the SARS–CoV–2 clinical isolate, which could be due to high dilution of the samples, which was necessary in order to prevent cell toxicity induced by human milk. However, the sometimes conflicting results (neutralization capacity of three HPP treated milk samples and not of the same unpasteurized milk samples) in the neutralizing test using the clinical isolate demonstrates a lack of robustness for this test in our setting. It seems unlikely that the HPP procedure adds neutralizing capacity. Another factor that has to be taken into ac–count as an explanation for the different results from these neutralization assays, is that the clinical isolate assay tests replication inhibition of SARS–COV–2, in contrast to the pseudovirus assay tests the prevention of SARS–CoV–2 infection.

Our data clearly indicate strong variability in an individual's antibody levels in response to COVID–19 infection. Some subjects show a more N protein or RBD directed antibody

response, and some exhibit a stronger IgA or IgG response. The presence and abundance of SARS–CoV–2 specific antibodies is known to be variable, for in–stance, the IgG response to the N protein is believed to occur earlier than the response to the S protein, but the titers are generally lower to the N protein compared to the S protein [24]. Also, in general IgM and IgA–class responses often occur earlier following disease onset, while IgG responses occur later and seem to be longer lasting [25]. Together, these results imply that testing for the presence of antibodies should not be directed against a single viral protein or focus on a single antibody class, but that different proteins and classes should be targeted to obtain the most complete and reliable information.

Donor milk banks around the world use HoP as a way to provide donor milk to preterm and sick term infants. However, HoP is known to affect the immune protection provided by human milk, due to the heat load which the milk is exposed. One promising alternative to HoP is HPP. HPP is already widely used in food industries as a non–thermal food preservation method that provides microbiologically safe products, while at the same time reducing the heat–induced damage of regular thermal pasteurization. Recent studies on HPP of donor human milk indicate that this method is capable of retaining significantly higher levels of antibodies when compared to HoP, while at the same time successfully eliminating microbes and viruses such as HIV and CMV [15,16,26–28]. Our data indicate that HPP would be a more suitable method to make human milk safe over thermal pasteurization, as indicated by the retention of functional IgAs in human milk, and the retained neutralization towards the clinical SARS–CoV–2 isolate.

This study has several strengths. First, we were able to collect clinical data, blood and human milk from almost all participants. Second, by using different methods to measure antibodies in serum and human milk we created a robust dataset, capturing the variation in antibody responses. As we included women with varying time frames between the onset of COVID–19 symptoms and collecting samples, we were able to investigate antibody dynamics within each individual subject. Moreover, by using virus neutralizing as a functional readout, we were able to draw conclusions on the effectiveness of the antibodies against the virus.

Human milk is known to be a safe product that can be used for preventive strategies, especially compared to pharmaceutical interventions (either medication or vaccination), and no detrimental side effects are to be expected from its intake. In a recent preprint study, it was determined that the virus itself is not present in human milk and that even if there is detected virus on the breast skin, viral contamination is effectively removed by cleaning the breast before pumping [29]. With breast cleaning and pumping strategies and by pasteurizing human milk it is possible to provide a safe product for donor human milk banks to use for vulnerable populations. Furthermore, neutralizing antibodies against the SARS–CoV–2 virus could be extracted from human milk and used as a highly targeted COVID–19 therapeutic.

However, using human milk as a preventive strategy requires ample availability of human milk from COVID–19 recovered women. While rates of seroprevalence of anti–SARS–CoV–2 IgG antibodies in the general population varies widely, “milk prevalence” rates in pregnant and lactating women are not known and might differ substantially among mothers from region to region.

The possibility to purify IgA from human milk from mothers who have recovered from COVID–19 also opens possibilities, but again requires availability of sufficient seroconverted milk. Currently major issues with obtaining donated milk, includes limitations of donors to visit

milk banking facilities, many of which are associated with hospitals, of which the general population has limited access [30]. Even though we are still far away from clinical applications, efforts should be undertaken to investigate the possibility of using human milk antibodies as a preventive strategy against SARS–CoV–2 infection and subsequent spread.

7.5. Conclusions

Human milk of mothers who were previously infected with SARS–CoV–2 contained significant amounts of IgA against SARS–CoV–2 for at least 13 weeks after the onset of symptoms. After pasteurization, total IgA antibody levels were affected by HoP, while SARS–CoV–2 specific antibody levels were affected by HPP. Human milk samples of several donors had neutralization capacity against a pseudovirus of SARS–CoV–2, which remained after non–thermal pasteurization. No correlation was observed between hu–man milk antibody levels and neutralization capacity.

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Institutional Review Board Statement
The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Commit–tee) of the Medical Ethics Committee of Amsterdam UMC, location VUmc (protocol code 2020.199—NL73686.029.20 and date of approval: 17 April 2020). Written informed consent was obtained from all participants.

Informed Consent Statement
Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

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Conflicts of Interest
The authors declare no conflict of interest.

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Supplementary Appendix

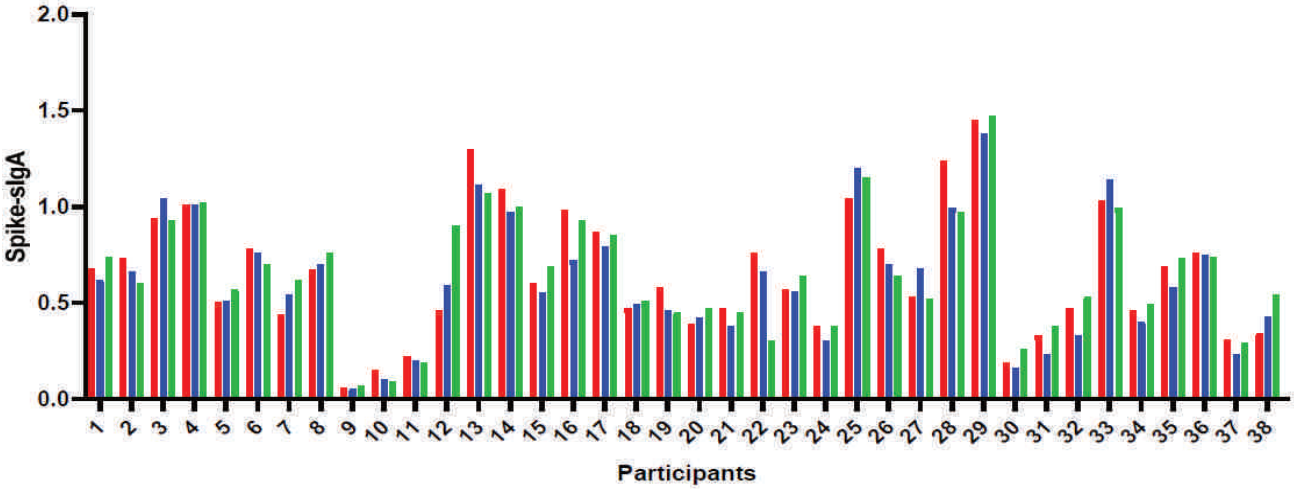

Figure S1. Spike IgA in human milk. Individual Spike IgA levels in UP milk (red), HPP milk (blue) and HoP milk (green).

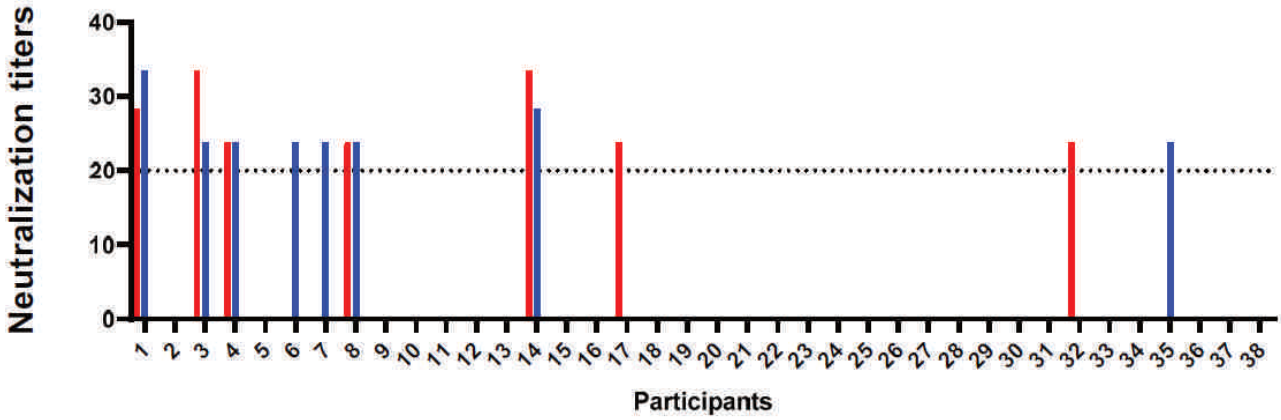

Figure S2. SARS–CoV–2 neutralizing capacity of human milk of a clinical isolate. Neutralization capacity in individual participants of UP milk (red), HPP milk (blue) and HoP milk (green)

Chapter 8

Processing methods of donor human milk evaluated by a blood plasma clotting assay

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Abstract

Donor human milk is the first alternative for preterm infants when mother's own milk is not available. Most available human milk banking guidelines recommend classical holder pasteurization to ensure safety by eliminating potential infectious microorganisms. Processing by heat treatment, however, negatively affects functionality and availability of bioactive components naturally present in human milk. Here we compared the effect of five different processing methods on the ability of human milk to induce blood plasma clotting, which was recently described as a bioactive function present in human milk. From thirty lactating women, milk samples were collected, and all milk samples were subjected to holder pasteurization (30 min at 62.5 °C), high–temperature–short–time pasteurization (15 s at 72 °C), high–pressure processing (5 min at 500 MPa), ultraviolet–C irradiation (4863 J/L), or thermo–ultrasonication (6 min at 60 W, at 40 °C). All methods significantly reduced the ability of milk to trigger blood plasma clotting compared to untreated milk, but ultraviolet–C irradiation and high–pressure processing were best at preserving this activity. Taken together, measuring the ability of milk to induce blood plasma clotting may offer a new tool to monitor the effect of human milk processing

Keywords: blood plasma clotting; coagulation; high–pressure processing; high–temperature–short–time; human milk; pasteurization; thermoultrasonication; ultraviolet–C irradiation

8.1. Introduction

Mother's own milk is considered the best diet for almost all newborns. Especially preterm infants may benefit from human milk as it is for example related to less intestinal complications such as necrotizing enterocolitis when compared to preterm infant formula feeding [1]. The reason human milk contributes to the reduced risk of (intestinal) inflammation is the presence of bioactive substances in human milk [2]. However, sometimes mother's own milk is insufficient or unavailable to preterm newborn infants. The next best choice for these infants is donor human milk [3]. Although milk donors are screened for health status, consumption of donated raw milk can still impose a serious health risk to preterm infants due to potential transmission of pathogens, mainly bacteria, cytomegalovirus and human immunodeficiency virus.

Considering these biosafety concerns, almost all human donor milk banks employ holder pasteurization (HoP) before the milk is provided to preterm infants [4]. Although HoP effectively eliminates pathogens, it also destroys or reduces (the functionality of) bioactive components present in milk. Therefore, at present there is a quest for alternative milk processing methods that are less harmful to bioactive components [5, 6].

Recently, we demonstrated that human milk, but not bovine, induces blood plasma clotting, although its precise biological role is uncertain. The ability of milk to induce blood plasma clotting may contribute to the relative lower incidence of necrotizing enterocolitis in preterm infants fed own mothers' milk when compared to those who are fed cow's milk based preterm formula [1]. The ability of human milk to induce blood plasma clotting is due to the presence of cell–derived particles called extracellular vesicles (EVs) [7]. EVs are small in size, about 50 nm to 1 μm, and are released by all cells into their environment. EVs are enclosed by

a phospholipid membrane, and are thought to play various roles in homeostasis, protection, and intercellular communication, the latter by transporting for example genetic information between cells [8]. The protein that triggers coagulation, a transmembrane protein called tissue factor (TF), is abundantly present in human milk, where it is exclusively present in the phospholipid membrane of EVs [7]

Also other human body fluids such as saliva contain TF–exposing EVs that induce blood plasma clotting [9]. Although it is unknown why such body fluids contain TF–exposing EVs, their presence in saliva has been associated with the reflex of licking a wound, i.e. to promote haemostasis and wound healing, thereby reducing the risk of developing infection. To which extent the EVs in milk may also promote haemostasis, e.g. during nipple skin damage or upon gastrointestinal damage in young infants, is yet unknown. Recently, we observed that TF–exposing EVs from normal human saliva and milk expose not only TF but the complex of TF and its ligand, coagulation factor VII (data not shown), which supports our hypothesis that TF–exposing EVs in milk may support haemostasis. The fact that already in the 1930s paediatricians demonstrated that nose bleeds of haemophilia patients could be effectively treated by gauzes soaked in human milk, directly proves that the ability of human milk to trigger blood clotting is indeed sufficient and effective in promoting haemostasis[10, 11].

The aim of the study was to determine the effect of five different milk processing methods compared to untreated milk on the ability of human milk to induce blood plasma clotting.

8.2. Methods

8.2.1. Sample collection

Lactating mothers were recruited in Amsterdam University Medical Center, Amsterdam, The Netherlands. Written informed consent was obtained from all participants. The study protocol was approved by the local Medical Ethical Committee. Milk was collected by complete expression of all milk in one breast, and milk samples were collected in disposable bisphenol A–free bottles by using a breast pump. The milk was stored at −20 °C. If the milk was collected at home, samples were stored at −20 °C in their home freezer, then transported on dry ice to the hospital.

8.2.2. Processing methods

To thaw the frozen milk, the bottle was put in a refrigerator overnight at 4 °C. Milk samples were split into 6 aliquots after which one baseline sample was stored untreated and the other aliquots underwent one out of five different processing methods: HoP (30 min at 62.5 °C), high–temperature–short–time pasteurization (HTST; 15 s at 72 °C), high–pressure processing (HPP; 5 min at 500 MPa), ultraviolet–C irradiation (UVC; 4863 J/L), or thermoultrasonication (TUS; 6 min at 60 W, 40 °C). The choice of milk processing methods was based on a review [12], and the specific conditions for HPP, HTST, UVC irradiation and TUS were derived from studies performed at the Wageningen University [13, 14].

For HoP, 30 mL milk from each donor was poured into 50 mL sterile Greiner tubes. The tubes were placed in a shaking water bath at 150 rotations per minute (rpm), where they were heated for 30 min at 62.5 °C. Subsequently all tubes were placed in an ice bath for 15 min

for cooling to <4 °C. The temperature was continuously monitored using a temperature data logger RS PRO 1384 (RS Components B.V., The Netherlands).

For HTST, a laboratory scale pasteurizer was built. The system includes a peristaltic pump (Watson Marlow 505S), two water baths representing the heating and the holding section, and an ice bath representing the cooling section. Milk samples of 10 mL from each donor were heated for 15 s at 72 °C, after which they were cooled to <10 °C.

For HPP, milk samples of 10 mL from each donor were poured into sterile pouches and subjected to 500 MPa for 5 min using a pilot scale high pressure pasteurizer (Resato, the Netherlands). This HPP pasteurizer used water as the pressure transmission medium.

For UVC irradiation, we used an experimental set up that was based on a previously published approach [15]. Milk samples of 60 mL from each donor were poured into sterile glass beakers of 150 mL. A UVC germicidal lamp (PL–S 5 W, UVC radiation 1.1 W, Philips, The Netherlands) was used as the source of UVC irradiation and was positioned diagonally in the beaker to ensure a vortical flow. The samples were stirred on a magnetic stirring plate at 500 rpm, with a 4 × 20 mm stirring rod (IKA RH basic 2, Germany) during the whole process. The approximate UVC dose was 4863 J/L.

For TUS, a sonifier (Branson Digital Sonifier 450, 50–60 Hz) that was fitted with a sound enclosure (Branson Emerson Technologies, GmbH & Co) and a microtip probe was used. The sonifier was then connected to a circulating water bath and milk samples of 20 mL from each donor were transferred into a glass beaker surrounded by circulating water of 40 °C. All experiments were performed at an amplitude of 58%, which produced an output of 60 W, as displayed by the instrument. The sonifier was set on a pulse–pause mode with a continuous pulse of 59.9 s and a pause of 20 s for a treatment time of 6 min. After treatment, the samples were cooled in an ice–water bath to 4 °C. A data logger (RS PRO 1384) was used to monitor the temperature at all times.

After all treatments, the samples were stored at −20 °C until analysis.

8.2.3. Blood collection and blood plasma preparation

To prepare a pool of normal human blood plasma, citrate–anticoagulated blood was collected from about 400 healthy subjects with informed consent by venepuncture through a 21–gauge needle using Vacutainer tubes (BD, Mississauga, Canada). The first 2 mL of blood was discarded to exclude contamination from venepuncture. The collected blood was centrifuged for 5 min at 4190 g at 18 °C to separate blood cells from the blood plasma. Subsequently, blood plasma was collected and transferred into a new tube, which was centrifuged for 15 min at 3000 g at 15 °C to remove most of the remaining blood platelets. After collection of the platelet–depleted blood plasma, the blood plasma was pooled, mixed, and stored as 500 µL aliquots at −80 °C until use.

8.2.4. Blood plasma clotting assay

To monitor the effect of processing methods on the ability of milk to trigger blood plasma clotting, blood plasma was thawed at 37 °C, followed by centrifugation at 18,900 g at 4 °C for one hour to remove residual cell fragments and debris as described earlier [7]. A schematic overview of the blood plasma clotting assay is shown in **Figure 1**. Human milk samples were thawed at 37 °C and then immediately placed on melting ice until use. In preliminary

experiments, we made milk titration curves (0.04% to 20% (vol/vol)) in blood plasma to find an optimal milk concentration to trigger clotting (**Figure 2**), and we arbitrarily chose 5% milk (vol/vol) as the optimal concentration. To obtain a final concentration of milk of approximately 5% (vol/vol), milk (50 µL) was diluted in 150 µL saline and gently mixed to ensure homogeneity. From this four–fold diluted milk sample, 20 µL was added to 70 µL blood plasma in a flat bottom 96 wells plate and mixed. In this mixture the milk provides the EV–bound TF, the initiator of clotting, and the blood plasma provides the coagulation factors. Because calcium ions are required to allow binding of coagulation factors to the EV membrane, and because calcium ions are chelated by the anticoagulant citrate to inhibit coagulation, CaCl2 was added (15 µL CaCl2 (100 mmol/L stock)) to allow blood plasma clot formation. After mixing, clotting was monitored for one hour at 37 °C by measuring the optical density at λ = 405 nm using a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA).

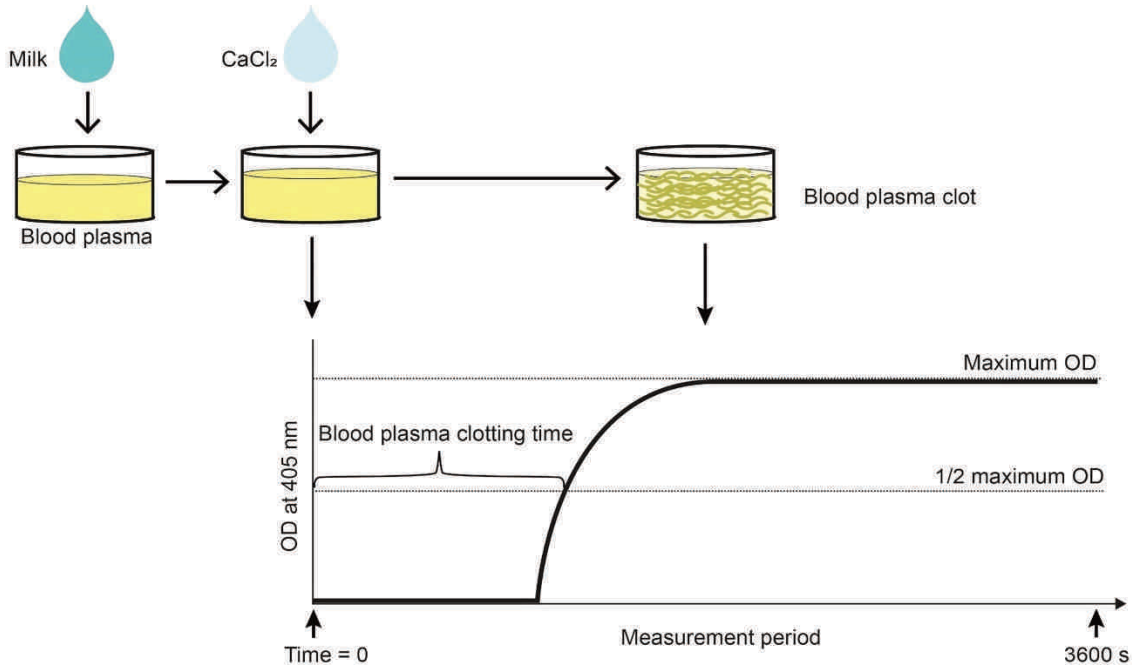

Figure 1. Principle of the blood plasma clotting assay. Human milk is added to blood plasma, mixed, and pre–heated at 37 °C. To initiate blood plasma clotting, CaCl2 is added (t = 0). Clot formation is recorded by monitoring the optical density (OD) of blood plasma for one hour at 37 °C. When the blood plasma clotting starts, the OD increases. The clotting time is automatically recorded as the ½ maximum OD.

8.2.5. Statistics

The blood plasma clotting time, as a measure for the presence TF, the initiator of coagulation, was analyzed using a Wilcoxon signed rank test (SPSS version 26.0 software, SPSS Inc., Chicago, IL). A probability value (P) of less than 0.05 was considered to be statistically significant. All data are shown as median and interquartile range, unless indicated otherwise.

8.3. Results

8.3.1. Baseline of donors

Milk samples were collected from 30 healthy women at median 4 months (range 2 weeks – 13 months) postpartum.

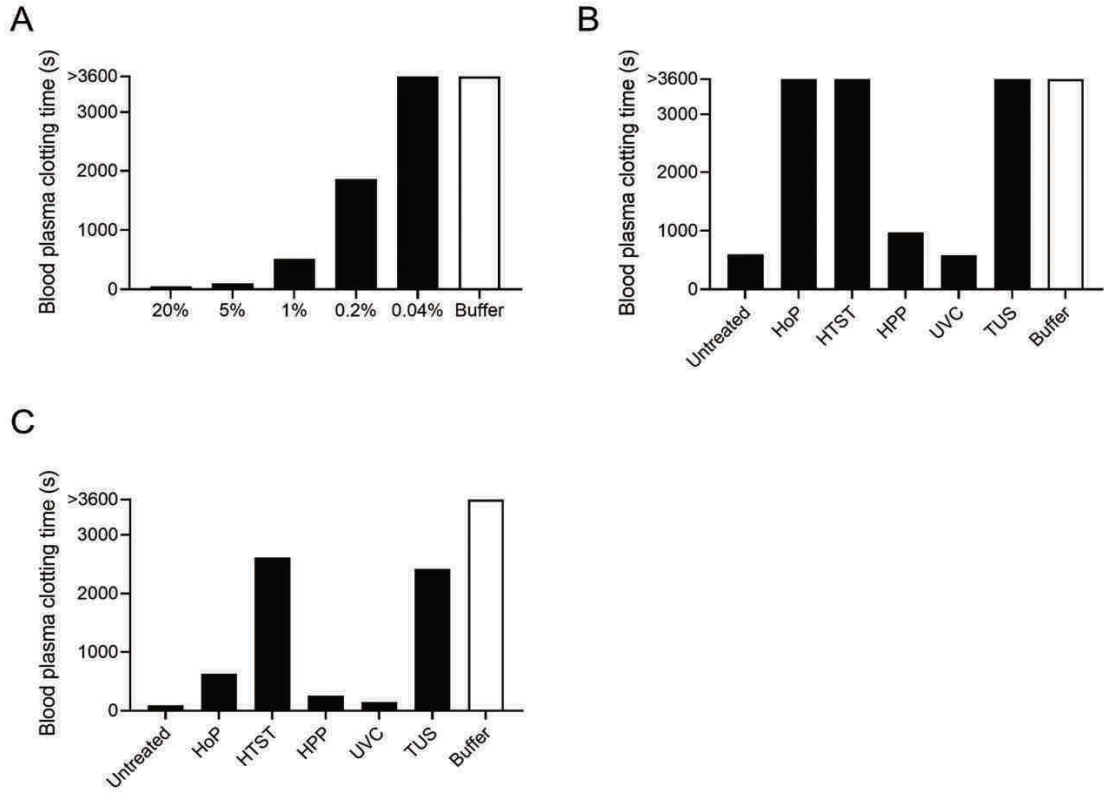

Figure 2. Finding the optimal concentration of human milk in a blood plasma clotting assay. Dilutions of human milk from a single donor were added to human plasma to find the optimal dilution (A). Based on the results from (A), the blood plasma clotting times were measured in the presence of 1% (B) or 5% (C) of untreated and processed milk from the same donor (vol/vol). Saline was used as negative control. HoP: holder pasteurization; HPP: high–pressure processing; HTST: high–temperature–short–time pasteurization; TUS: thermoultrasonication; UVC: ultraviolet–C irradiation

8.3.2. Effect of processing methods on the ability of human milk to induce blood plasma clotting

In preliminary experiments, we diluted unprocessed human milk from one donor to measure its ability to trigger blood plasma clotting. From **Figure 2A** it is clear that the ability of milk to trigger blood plasma clotting is concentration dependent. For example, addition of 20% (5–fold dilution) milk (vol/vol) resulted in almost immediate blood plasma clotting, whereas at a final dilution of 0.2% (500–fold dilution) the blood plasma clotting was hardly recordable within one hour. Therefore, we tested 1% and 5% final concentrations of milk in blood plasma (vol/vol) for all processing methods studied. As shown in **Figures 2B** (1%) and **2C** (5%), at a final concentration of 5%, the blood plasma clotting time of all processing methods was within the detection range, and therefore this concentration was chosen to study the clotting ability of untreated and processed milk samples from 30 donors.

Figure 3 shows that after addition of 5% (vol/vol) untreated human milk (n = 30) the blood plasma clotting time was 68 s (median; interquartile range 34–99). Compared to untreated milk, treatment of milk samples with HoP increased the blood plasma clotting time to 1259 s (686–3156; $P < 0.001$), whereas TUS and HTST increased the blood plasma clotting time to 1256 s (512–3276) and 809 s (564–1189), respectively (both $P < 0.001$). Treatment of milk

with HPP modestly affected the ability of human milk to induce blood plasma clotting, with a blood plasma clotting time of 239 s (133–315; P < 0.001). UVC, however, preserved the ability of human milk to induce blood plasma clotting best from all tested processing methods with a blood plasma clotting time of 92 s (44–150), although this is still significantly prolonged compared to untreated milk (P = 0.033). Also, UVC preserved clotting activity significantly better than HPP (P < 0.001). **Supplementary Table 1** shows the blood plasma clotting times of all individual donors.

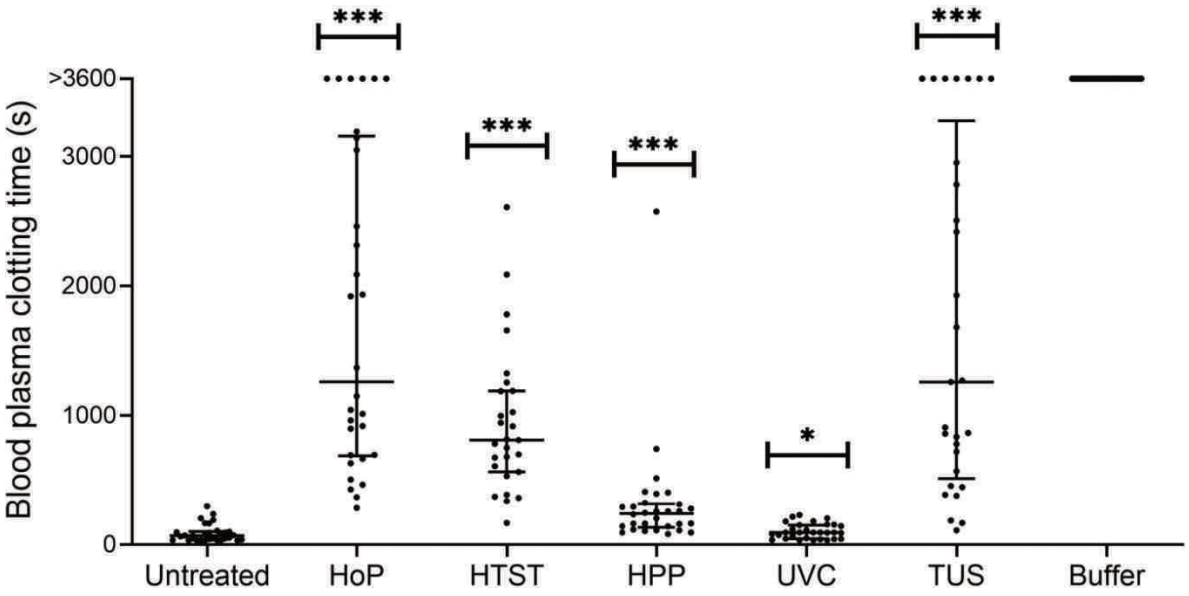

Figure 3. Effect of milk processing methods on blood plasma clotting. Human milk was collected from 30 healthy lactating women. The clotting of blood plasma was measured for one hour, i.e. 3600 s (s), in the presence of 5% untreated or processed milk (vol/vol). Buffer (saline) was used as negative control (n = 30). HoP: holder pasteurization; HPP: high–pressure processing; HTST: high–temperature–short–time pasteurization; TUS: thermo–ultrasonication; UVC: ultraviolet–C irradiation; *P < 0.05; ***P < 0.001.

8.4. Discussion

In the present study, we found that non–thermal methods preserve the blood plasma clotting ability of human milk better than thermal methods. Recently, we showed that human milk induces blood plasma clotting, and this milk activity is sensitive to HoP [7]. In the present study, we confirm these findings. In addition, HTST, a processing method that reduces thermal damage of bioactive components in human milk, also impairs the ability of human milk to induce blood plasma clotting, indicating that heating–based processing methods are not optimal to preserve this bioactive function [16]. Also TUS strongly affected the ability of milk to induce clotting. TUS–created cavitation kills microorganisms by mechanical and sonochemical damage, and this method may in the same way also damage EVs, possibly explaining the failure of a subset of milk samples to induce blood plasma clotting [17].

In our present study, three milk processing methods involve a heating step, i.e. HoP, HTST, and TUS. These thermal processing methods are known to reduce the levels of native immune–active proteins and enzymes [18] and all three methods reduce the ability of TF to trigger blood plasma clotting. Also HPP may induce irreversible structural changes of proteins

and enzymes present in milk [19], but such changes are not expected to occur at the mild conditions that were applied in the present study.

In contrast, HPP is better at preserving bioactive components as immunoglobulins and growth factors than HoP and HTST [20], and in the present study we detected a modest effect of HPP on the ability of milk to induce blood plasma clotting. HPP destroys the tertiary structure of proteins, thereby impairing the microbial internal biochemistry, but HPP only seems to modestly affect the ability of milk to induce blood plasma clotting, which may be due to the fact that we applied relatively mild HPP conditions. UVC is a gentle and safe alternative for human milk processing as this method preserves more bioactive factors than HoP and HTST [21, 22]. From our present results, UVC hardly affects the ability of human milk to induce blood plasma clotting, and thus UVC seems best at preserving the structure and function of EV–exposed TF.

From **Figure 3** it is clear that the variation in blood plasma clotting times is larger for thermal processing methods than for HPP and UVC. When sufficient functional TF is added to blood plasma, the blood plasma clotting reaction (which is an exponential reaction) will start immediately. When TF is absent (as in the buffer condition), or when functional TF is present at a low concentration (after heat treatment), the blood plasma will not clot or will clot late. Thus, because the concentration of functional TF is a critical factor in the blood clotting reaction, the observed variation in blood plasma clotting times is less for methods that only modestly affect the functional activity of TF.

Limitations of the present study are that a contribution of TF from non–EV origin cannot be excluded because whole milk was stored, and experiments were not performed in duplicate, although in a preliminary experiment the variation was <15% (**Supplementary Figure 1**).

In conclusion, we found that UVC and HPP, gentle milk processing methods both known to preserve bioactive components, hardly affect the ability of human milk to trigger blood plasma clotting. In contrast, HoP, HTST, and TUS, processing methods that are not optimal for preserving bioactive components of milk, all three impaired the ability of milk to induce blood plasma clotting.

To which extent measuring blood plasma clotting is an alternative, additional and/or superior bioassay to monitor the effect of donor human milk processing methods, and to which extent the results from this assay are associated with clinical outcome, clearly requires head–to–head comparisons and additional studies. Nevertheless, the blood plasma clotting assay is easy, fast and robust, and may be included to future milk processing studies to investigate its potential clinical relevance. Ultimately, if proven safe, this should lead to the routine implementation of more gentle processing methods of donor human milk. This could then improve its clinical benefits for preterm infants, in case mother's own milk is insufficient.

Declaration of Competing Interest

C.H.P.v.d.A. reports participating in scientific advisory boards and giving lectures in educational symposia for Nutricia Early Life Nutrition, Baxter, and Nestlé Nutritional Institute. J.B.v.G. is member of the National Health Council and founder and director of the National Human Donor Milk Bank in the Netherlands.

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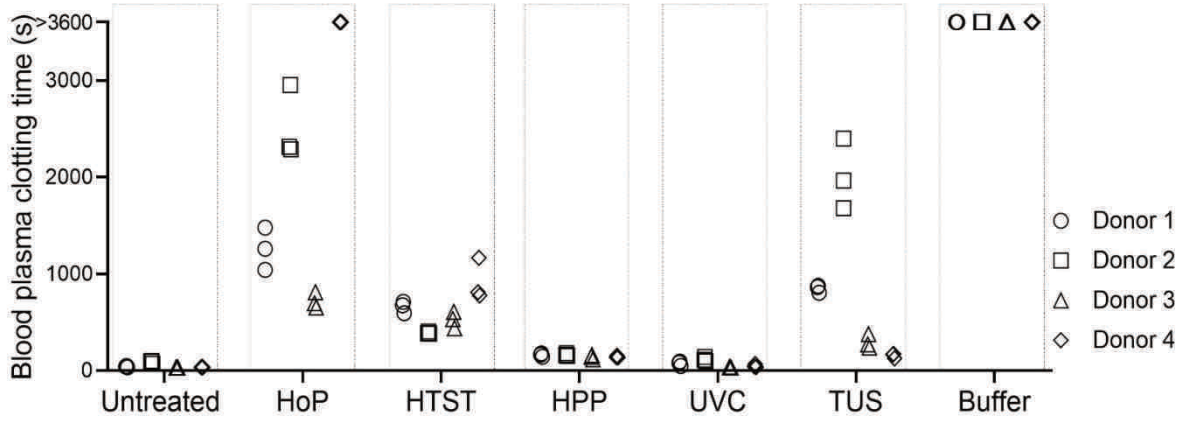
Supplementary Appendix

Supplemental Table 1. Effect of milk processing methods on blood plasma clotting.

	Blood plasma clotting time (s)						
Donor	Untreated	HoP	HTST	HPP	UVC	TUS	Buffer
1	41	1041	674	164	80	864	3600
2	69	2313	385	162	112	1679	3600
3	33	694	531	115	34	378	3600
4	34	3600	809	139	41	167	3600
5	94	3600	1656	408	93	568	3600
6	76	897	1025	162	45	446	3600
7	32	917	337	109	67	—	3600
8	165	3600	699	291	202	1269	3600
9	97	630	2610	257	149	2419	3600
10	203	1368	564	403	153	3600	3600
11	297	3051	1323	257	90	833	3600
12	93	1918	814	159	214	455	3600
13	67	3192	1187	278	48	779	3600
14	235	2461	917	93	93	186	3600
15	71	3600	1252	2575	155	2783	3600
16	46	1149	679	204	92	907	3600
17	189	3600	942	741	181	2507	3600
18	80	3144	1780	258	143	3600	3600
19	162	1932	996	394	178	3600	3600
20	52	2087	781	312	43	859	3600
21	33	693	—	103	35	109	3600
22	90	427	—	293	92	3600	3600
23	54	3600	1189	324	93	1926	3600
24	32	664	371	80	33	720	3600
25	31	367	606	234	39	385	3600
26	32	962	166	92	88	3600	3600
27	38	284	—	244	73	3600	3600
28	62	464	2086	514	227	3600	3600
29	33	1012	361	142	33	1256	3600
30	105	505	749	109	118	2953	3600



Supplementary Figure 1. Reproducibility of blood plasma clotting assay. Human milk samples from randomly selected donors (n=4) were added to human blood plasma at the final concentration of 5 % (vol/vol) in triplicate. Saline was used as a negative control. HoP: holder pasteurization; HPP: high–pressure processing; HTST: high–temperature–short–time pasteurization; TUS: thermoultrasonication; UVC: ultraviolet–C irradiation. For each donor, a different symbol is used.

Chapter 9

General Discussion

Human milk banks (HMBs) apply holder pasteurization (HoP) to donor human milk (DHM) before its provision to high–risk infants, since this method efficiently eliminates most microbial contaminants and preserves important nutritional components. However, bioactive components that play a crucial role in the infants' optimal development and growth are significantly decreased [1]. On this account, the current thesis aimed to evaluate the effects of thermal and non–thermal techniques as alternatives to HoP on the safety of DHM and the preservation of its bioactivity. The current chapter reflects upon these findings and provides recommendations for future research.

9.1. Selection of the most suitable processing method

As defined by the European milk banking association (EMBA), a novel processing method should preserve the DHM's bioactivity better than HoP, while ensuring microbiological safety [2]. These new methods should be of low cost, easily implementable, tested both at lab and HMB settings, with available detailed equipment description, process repeatability and control [2]. High–temperature–short–time (HTST), high–pressure processing (HPP), ultraviolet–C irradiation (UV–C) and thermoultrasonication (TUS) were the processing methods selected to be investigated in the current thesis, as they seem to be the most promising alternatives to HoP based on the available literature at the start of this PhD period [2–4].

9.1.1. Safety

As previously discussed, safety is the first parameter that needs to be assessed when evaluating a processing method. Based on the human milk banking guidelines, a suitable method for DHM processing should be able to achieve at least a 5–$\log_{10}$ bacterial reduction [5–7]. As EMBA additionally states, the possibility of spore inactivation with a novel method is considered a great advantage over HoP, while at the same time, inactivation of viruses than can be transmitted through breastfeeding should be further assessed with these methods as well [6]. A detailed description of the techniques available for both bacterial and viral inactivation assessments can be found in **chapter 2**.

9.1.1.1. Bacteria inactivation with thermal and non–thermal alternatives

All thermal treatments studied in this thesis resulted in a negative (alkaline phosphatase) ALP test, which served as a marker for pasteurization efficacy (**chapter 4**). Our findings agree with previous reports that showed a complete inactivation of ALP after HoP [8]. The effectiveness of HTST and flash heating (FH) in bacterial inactivation has been further demonstrated with a number of bacterial strains [9–14], as shown in **Table 1**.

Since ALP is a heat load marker which is not inactivated with non–thermal methods, microbiological safety with such methods was assessed by the artificial contamination of untreated samples with clinically relevant bacteria (10^8 CFU/mL) and their counts after processing (**chapter 5**). It was shown that HPP, TUS and the highest UV–C dosage tested (4863 J/L) resulted in a >5–$\log_{10}$ reduction of *E. cloacae*, *S. epidermidis* and *E. coli* K12 counts. Complete microbial inactivation was also documented after HoP. Specifically for HPP, several other studies support its efficacy in achieving DHM microbiological safety (**Table 2**).

Table 1. Summary of studies investigating bacterial inactivation in DHM after HTST and FH.

Treatment parameters	Equipment	Bacterial strains	Inoculation level (CFU/mL)	Reduction	Reference
HTST (72 °C–15 sec)	Continuous–flow HTST pasteurizer	*S. agalactiae* CIVO.B.0062	10^6	6–$\log_{10}$	[11]
		S. aureus NCCB70054	10^7	Undetectable counts	
		E. coli CIVO.B.0505	10^8		
		S. aureus ATCC 25923	10^7	>6–$\log_{10}$	[12]
		E. coli ATCC 25922	10^6	>5–$\log_{10}$	
		Total background flora	Mean bacterial counts: 4.16, 3.97, 3.55, 2.45, 2.65 and 5.14–$\log_{10}$, respectively for each strain	Only *B. cereus* survived (2.43−$\log_{10}$)	[15]
	Benchtop continuous–flow HTST pasteurizer	*L. monocytogenes* ATCC 7644	10^6	≥6–$\log_{10}$	[9]
		C. sakazakii ATCC 51329	10^6	≥6–$\log_{10}$	
		S. aureus ATCC 33862	10^6	≥6–$\log_{10}$	
		Total background flora	Total counts $<10^5$, *S. aureus* & *Enterobacteriaceae* $<10^4$	Undetectable counts	[16]
	Thin–layer HTST device	*S. aureus* ATCC 6538	10^4	4–$\log_{10}$	[17]
		E. faecalis ATCC 29212	10^5	4–log10	
		K. pneumoniae ATCC 700603	10^4	>4.8–$\log_{10}$	
		K. pneumoniae Kpn 01605	10^4	>4.6–$\log_{10}$	
	Injection to an industrial plate heat exchanger through a water stream	Total background flora	760 ±970 CFU/mL (Mean ± standard deviation)	Undetectable counts	[10]
FH	Aluminum pan with water on a single–burner butane stove	*S. aureus, E. coli*	10^6	>5–$\log_{10}$	[14]
		Total background flora	Total counts and *S.aureus* 10^3	Undetectable counts	[13]
	Beaker with water on a hot plate	Total background flora	Total counts $>10^7$	41% $<10^3$ counts, 59% $\geq 10^3$ counts	[18]

Studies comparing HPP to HoP in bacterial inactivation reported no significant differences [19–21], while HPP was found to be superior to HoP in the inactivation of sporulated *Bacillus* spp [22, 23]. Different degrees of resistance to inactivation by HPP have also been reported for different bacterial strains, which usually depends on the selected processing parameters (pressure, time and temperature) [23]. *S. aureus* ATCC 6538 is often used in such tests as a model bacterium due to its high resistance to HPP and when more intense conditions were used, successful elimination was reported [24–26].

Table 2. Summary of studies investigating bacterial inactivation in DHM after HPP.

Treatment	Parameters	Bacterial strains	Inoculation level (CFU/mL)	Reduction	Reference
HPP	500 MPa, 8 min	Total background flora	Total counts $>10^7$	71% $<10^3$ and 29% $\geq 10^3$ counts	[18]
	425 MPa, 4x6 min, 4ºC or 37 ºC		Total counts, 10^4–10^5	67%, undetectable counts. 33%, only *Bacillus spp* counts (<10–10^2)	[22]
	300MPa, 4x10 min, 40 ºC		Total counts (mainly sporulated *B. cereus*), 10^5	Undetectable counts	
	400, 450, 500 MPa, 5 min, 20°C		Aerobic bacteria 10^5–10^6, Total coliforms, 10^4–10^5	Undetectable counts	[21]
	300–600 MPa, 5, 10, 15 min, 25 ºC		Total counts $<10^5$	Undetectable counts after ≥ 400 MPa	[31]
	400, 500,600 MPa, 5 min, 12°C		Total counts, 10^2–10^4	Undetectable counts	[19]
	350 MPa, 4x5 min, 38 ºC	*S. aureus* ATCC 6538 / *B. cereus* (spores) ATCC 14579	10^6	6–$\log_{10}$	[26]
	500 MPa, 0–15min, 4, 20, 50°C	*S. aureus ATCC 6538*	10^8	>6–$\log_{10}$ after 10 min at 500 MPa and 50°C & 5–$\log_{10}$ after 15 min at 500 MPa and 4 or 20°C	[24]
		Total background flora	10^4	Significant reduction after 5 min at 300 MPa and 4°C	
		Enterobacteriaceae	Native contamination, 10^2	Total reduction already after 5 min at 300 MPa and 4°C	
	400 MPa, 0–50 min, 21–31ºC	*S. aureus* ATCC 6538	10^8	6–$\log_{10}$ 30 min at 400 MPa	[25]

Table 2. *Continued*

HPP		*S. aureus* ATCC 25923		8–log_{10} 30 min at 400 MPa	
		S. agalactiae ATCC 12927		8–log_{10} 4 min at 400 MPa	
		L. monocytogenes ATCC 19115		8–log_{10} 2 min at 400 MPa	
		E. coli ATCC 25922		6–log_{10} 30 min at 400 MPa	
	593.96 MPa, 233 sec	*S. aureus* CECT 976	10^7	5.81–log_{10}	[32]
		B. cereus CECT 131 (vegetative cells)		6.93–log_{10}	
	600 MPa, 200+400 MPa, 100+600 MPa, 200+ 600 MPa, each for10min, 21ºC	Total background flora	10^3	Undetectable counts	[33]

As shown in **Table 3**, there are limited studies available on the efficacy of UV–C to eliminate bacteria in DHM. The differences in the experimental set up of these studies make direct comparisons difficult, but the studies using similar UV–C settings and equipment to those described in this thesis were able to show a 5–log_{10} reduction of bacterial species, including *Bacillus* spp [27, 28]. Similarly, there are very limited data regarding TUS and bacteria inactivation in DHM (**Table 4**) [3]. However, studies with settings comparable to those of this thesis reported a 5–log_{10} bacterial reduction, both in DHM and in bovine milk [29, 30].

Apart from the selected treatment conditions, the variation often reported on the inactivation of certain bacteria species with non–thermal treatments may also be depended on the strain, growth medium and growth phase [37]. Microorganisms resistant to such treatments could potentially suffer only sublethal injuries and could be able to grow again under suitable conditions [38]. For instance, a study on the effect of HPP on *L. monocytogenes* suspended in citrate buffer showed that although the bacterium was completely inactivated, resuscitation was possible 2–4 days after the treatment [39]. Viazis and colleagues used nonselective and selective media in order to estimate both the healthy and the sublethally damaged cells in HPP–treated DHM, and did not find a high degree of sublethally damaged cells [25]. The authors considered the presence of antimicrobial compounds in the HPP–treated DHM as the explanation for why resuscitation was not observed [25]. It has also been reported that UV–C may induce a viable but not culturable (VBNC) state in certain bacteria, but data on whether such bacteria can still pose a threat to human health are currently lacking [40, 41]. However, Kramer and colleagues showed that such bacteria were still unable to express proteins, which suggests that they might not pose a health risk [41]. Limited data exist for the possibility of TUS to induce a VBNC state as well,

but a treatment of 15 min at 55°C and 100W was shown to reduce the number of sublethally damaged *S. aureus* cells to negligible numbers [42].

Table 3. Summary of studies investigating bacterial inactivation in DHM after UV–C.

Treatment	Parameters	Bacterial strains	Inoculation level (CFU/mL)	Reduction	Reference
UV–C	85–740 J/L	*S aureus PCM 2054*	10^5	5–$\log_{10}$, 400 J/L	[34]
		E. coli K12		5–$\log_{10}$, 700 J/L	
		E. faecalis PCM 896		2.9–$\log_{10}$, 740 J/L	
		E. faecium ATCC 6057		3.95–$\log_{10}$, 740 J/L	
	250 nm, 25 min	Total background flora	Total counts >10^7	35% of the samples had <10^3 counts and 65% ≥10^3 counts	[18]
	4863 J/L	*E. coli*, *S. epidermidis, E. cloacae*, *B. cereus* and *S. aureus*	10^5	5–$\log_{10}$	[27]
	4863 J/L	Total background flora	Total counts (aerobes & *Enterobacteriaceae*), 10^5	5–$\log_{10}$	[28]
	550, 2750, 8250, 16300, 33000 J/L (254 nm)	*C. sakazakii* BAA–894	10^8	>5–$\log_{10}$, ≥8250 J/L	[35]
		E. faecium ATCC 8459			
		S. aureus 138–, 146–CPS			
		Spores: *B. subtilis* NRRL B–354, 356, *P. polymyxa* B–510, *P. macerans* B–14029. Cocktail: *L. monocytogenes* ATCC 19115			

Due to safety issues in our university setting, most tests in this thesis were performed with biosafety level 1 strains. However, since the UV–C set up was placed in a biosafety level 2 laboratory, additional testing with pathogenic strains was allowed. After estimating bacterial inactivation, their outgrowth and resuscitation was assessed by incubating the DHM samples at room temperature for 5 and 24h (**Table 5**). HPP– and TUS–treated samples showed no bacterial outgrowth while some outgrowth was observed after UV–C treatment, but the increase in bacterial counts was not significant ($p>0.05$). UV–C treated samples were additionally incubated at 37°C for 5h and no significant outgrowth was observed. Outgrowth was not observed even when the HPP–treated samples were additionally incubated at 37°C for 24h and at room temperature for 3 more days.

Table 4. Summary of studies investigating bacterial inactivation in DHM after UV–C and TUS.

Treatment	Parameters	Bacterial strains	Inoculation level (CFU/mL)	Reduction	Reference
TUS	60W, 0–10min, 45 and 50 ºC	*E. coli* K12 ATCC 1498	10^5	>5–$\log_{10}$ after 4 min at 45 ºC and after 2 min at 50 ºC	[29]
		S. epidermidis ATCC 1498			
	100W, 4min, 60°C (ultrasound intensity: 1591 mW/cm^2)	*S. aureus* ATCC 6538	10^7	5.6–$\log_{10}$	[36]
		E. coli ATCC 10536		5.4–$\log_{10}$	
		Salmonella spp ATCC 14028		3.7–$\log_{10}$	

The conclusions of this thesis regarding the outgrowth and resuscitation of the stressed bacterial cells were based on culture–depended techniques [43]. Alternative techniques and advanced culture–based method have been proposed as more accurate for the detection of viable and non–viable bacterial cells, which include staining of the bacterial cell membrane and flow cytometric analysis, direct fluorescent antibody methods and DNA–binding agents in combination with RT–PCR, among others [44]. To avoid false–positive or false–negative results, future studies investigating bacterial recovery should also measure certain viability indicators such as culturability and metabolic activity, as well as membrane integrity.

9.1.1.2. Viral inactivation

Before applying HoP to DHM, HMBs first conduct rigorous donor screening to ensure DHM's viral safety. This step usually includes interviews and serological testing for viruses such as HIV, hepatitis B and C [7, 45]. However, as discussed in **chapter 2**, variations with regard to CMV and HTLV serological screening exist among the different HMBs.

Heat treatment is broadly used for viral inactivation, since it effectively inactivates both enveloped and non–enveloped viruses [46]. Inactivation can be achieved through the heat–induced denaturation of viral proteins and the disruption of viral particles, which eventually leads to formations of non–infectious viral subunits [46]. HoP is highly effective against a number of viruses, while HTST has been found to be more effective against lipid–enveloped viruses than nonlipid–enveloped viruses, like hepatitis A (**Table 6**). The most important viruses are CMV, HTLV and HIV because of the risk of mother to infant transmission through HM and the risk of chronic infection [47]. According to EMBA, inactivation of such viruses should be assessed for each novel processing method and device designed for DHM treatment [2].

Viral inactivation after HPP, UV–C and TUS was not determined in this thesis. Only a limited number of studies evaluating the ability of such methods to inactivate viruses in DHM are available [62, 63]. The mechanism through which HPP inactivates viral pathogens is related to the disruption of the viral envelope and the blockage of virus particles from binding to cells, while UV–C inhibits viral replication by forming pyrimidine dimers in the viral genome [46, 64].

The level of inactivation achieved with HPP may be variable, due to the high structural diversity that exists among viruses [65]. The only study available in DHM, showed a total CMV

inactivation in HPP–treated DHM samples, after 350 MPa at 38 ºC and four cycles of 5 min [62]. The studies investigating HPP against human viruses are mostly performed on culture media, cell lines or non–milk matrices [18]. The complete inactivation of hepatitis A, herpes simplex virus type 1, HIV–1 and CMV at pressures of 300–450MPa for 5–10 min is reported in these studies, but some HIV strains were found to be more HPP–resistant and required pressures of 500 MPa for 60 min for their inactivation [66–69].

UV–C has been shown to successfully inactivate a number of viruses in air, water, various kinds of surfaces and solid foods [70, 71]. Viruses with more complex capsid structures may be more UV–C resistant, thus requiring higher dosages for their inactivation, but the data on opaque liquid matrices such as milk are generally limited [72]. To the author's knowledge, only one study has evaluated viral inactivation by UV–C in DHM, and showed that a dosage of 64 mJ/cm^2 eliminates the replicative capacity of CMV in DHM [63]. UV–C has also been shown to effectively inactivate cell culture–derived hepatitis C virus and model viruses in UV–C–treated skim milk, while dosages <4000J/L were found effective against a number of swine viruses in porcine and bovine plasma [46, 72–74].

TUS has mostly been tested against bacteria, although the substantially limited number of studies investigating ultrasonication against viruses report reduction in viral titers [75]. All in all, further testing with regards to the viral safety of non–thermal techniques is required, before such methods can be implemented in HMBs.

9.1.2. Functionality

HoP negatively affects a great number of the milk's bioactive components. In **chapter 4**, **5** and **6,** we confirmed the significant IgA, LTF, LYZ, BSSL and insulin losses reported in the literature for HoP–treated DHM. At the same time, the bacteriostatic properties of HoP–treated samples were significantly reduced, while the milk's procoagulant activity and the neutralization capacity against a SARS–CoV–2 pseudovirus were eliminated (**chapters 7** and **8**).

Although the effects of HoP in DHM's bioactivity are well–studied, the level of degradation of some components remains difficult to quantify [1]. For example, **Table 1** in **chapter 1** shows that the decrease in IgA levels after HoP varies from 17% to 60% in the literature. The different DHM volumes treated and the different devices used may explain such discrepancies [65]. Furthermore, when the performance of HoP among European HMBs was evaluated (**chapter 3**), the long heating up and cooling down phases reported by European HMBs were mainly attributed to the pasteurizer design and the DHM volume included within one pasteurization cycle. HoP is commonly performed in standard pasteurizers with either water or air as heating medium, and in shaking or stationary water baths [20, 76–78]. Older models may expose DHM longer to critical temperatures than new optimized ones, whereas air pasteurizers may offer less homogeneous treatments and larger plateau durations than water pasteurizers [79, 80]. These prolonged treatments have a negative impact on the DHM bioactive components, since denaturation of BSSL already takes place at 45°C, IgA, LTF and LYZ are significantly damaged at 58°C, while for every minute spent at 62.5°C, their levels are reduced by 1.6%, 2.4% and 1.7%, respectively [81–83]. In the current thesis, HoP was performed in a closed shaking water bath, while the whole process was monitored with thermocouples and a temperature datalogger. For a DHM volume of 100 mL, the total processing time was 73 min,

which is shorter than the time usually required in batch processes (99±30 min, mean±sd, **chapter 3**). Higher losses of DHM components may thus occur in a HMB setting, where volumes as high as 450 mL may be processed (**chapter 3**), leading to longer heating up times.

Table 5. Inactivation and outgrowth of selected bacteria in DHM samples after HPP, UV–C and TUS

Bacteria		Inoculated as	Treatment tested	Treatment	Inactivation	*Outgrowth at room temperature (log CFU/mL)*				*Outgrowth at 37 °C (log CFU/mL)*	
						(5h)		(24h)		(5h)	
						S	NS	S	NS		
S. epidermidis	ATCC, 14990	Single strain	**HPP, UV–C, TUS**	2430 J/L	5.00 ± 0.3	NO	NO	NO	NO	0.8±0.1	
				3645 J/L	5.95 ± 0.1				NO	0.8±0.0	
				4860 J/L	6.95 ± 0.4				0.4±0.2	0.9±0.1	
				HPP, TUS	> 6.0	NO				ND	
*S. aureus** (MRSA)	11MN 52GN 29GN	Cocktail	**UV–C**	2430 J/L	5.0±0.3	NO	NO	NO	NO	1.4±0.2	
				3645 J/L	6.2±0.1	0.02±0.01	NO	0.08±0.1	0.1±0.0	1.4±0.1	
				4860 J/L	7.1±0,8	0.01±0.01		NO	0.3±0.04	1.7±0.5	
E. coli K12	K12 (DSM498)	Single strain	**UV–C, TUS**	2430 J/L	4.4±0.1	NO	NO	0.71±0.2	0.66±0.0	2.2±0.2	
				3645 J/L	5.5±0.3			0.83±0.3	0.24±0.0	2.6±0.3	
				4860 J/L	7.0±0.1			0.43±0.2	0.41±0.0	2.6±0.2	
				TUS	> 6.4	NO				ND	
*E. coli** (pathogenic)	O177:H7 (EHEC) O157:H7 (EHEC) O159:H34 (ETEC) O159:H34 (ETEC)	Cocktail	**UV–C**	2430 J/L	3.7±0.3	NO	NO	NO	1.8±0.3	1.8±0.7	
				3645 J/L	4.8±0.5			0.1±0.2	1.5±0.1	1.9±0.8	
				4860 J/L	5.7±1.4			NO	1.7±0.01	2.2±0.0	
E. cloacae	ATCC, 13047	Single strain	**HPP, UV–C, TUS**	2430 J/L	4.3±0.0	NO	NO	0.8±0.2	0.9±0.2	1.3±0.1	
				3645 J/L	4.6±0.0			0.9±0.1	0.9±0.5	2.6±0.3	
				4860 J/L	5.8±0.2			1.1±0.4	1.0±0.2	2.8±0.9	
				HPP, TUS	> 6.3	NO				ND	

*Provided by RIVM (Rijksinstituut voor Volksgezondheid en Milieu), ATCC; American Type Culture Collection, MRSA; methicillin–resistant S. aureus, EHEC; enterohaemorrhagic E. coli, ETEC; enterotoxigenic E. coli, NO; no outgrowth, ND; not determined, S; selective media, NS; non–selective media

Table 6. Summary of studies investigating viral inactivation in DHM with heat treatment.

Viruses	Level of inactivation	**Heat treatment** (HoP, HTST, FH, other combinations)
Human immunodeficiency virus (HIV)	***Complete inactivation***	**HoP**, > 5–log, TCID50 assay [48] **HTST**, **>7**–log, TCID50 assay [11] **FH**, 99.7% cell death, TZM–bl assay [51] **FH**, ≥ 3–log, RT activity [52] **56°C–33 min**, 4–log, RT activity [49] **55–70°C–until max temperature**, 4–log, GFP indicator cells [50]
Human T–lymphotropic virus (HTLV)		**56°C–33 min or 90°C –10 min**, 100% reduction, RT [53]
Cytomegalovirus (CMV)		**HoP**, 100% reduction, SEAP Reporter [55] **HoP or HTST (72 °C–5 s)**, no IEA+ cells, EAIF[54] **HoP or HTST,** no IEA+ cells, EAIF [17] **HTST**(72 °C–5 s or 87°C–5 s), >5–log, PRA [10]
Hepatitis C virus		**56°C–40 min, 60°C–10 min or 65°C–4 min**, tested negative, FFU assay*****
Ebola virus		**HoP**, >5–log, PRA [56]
SARS–CoV–2		**HoP**, 100% reduction, TCID50 assay [57], [58]
Pseudorabies virus		**HTST**, **>**8–log, TCID50 assay [11]
Human papillomavirus		**HoP**, 100% reduction, SEAP Reporter [55]
Marburg virus		**HoP**, >5–log, PRA [56]
Bovine diarrhea virus		**HTST**, **>7**–log, TCID50 assay [11]
Zika virus		**HoP**, >6–log, TCID50 assay [59]
Herpes simplex virus–1		**HoP,** 4.2–log, PRA [60]
Hepatitis A virus	***Limited inactivation***	**HTST**, 2–log, TCID50 assay [11]
Hepatitis B virus		**HoP**,17–29% reduction in viral markers, DNA extraction and PCR assays [61]
Porcine parvovirus		**HTST**, <1–log, TCID50 assays [11]

GFP; green fluorescent protein, RT; reverse transcriptase, FFU; focus–forming unit, PRA; plaque reduction assay, SEAP; secreted embryonic alkaline phosphatase, EAIF; early antigen immunofluorescence.*The tests were performed in human serum.

Based on these findings, **chapter 4** was focused on whether shorter heating up times and shorter pasteurisation duration at a higher temperature could preserve the DHM bioactive components better. It was shown that HTST, the method with the shortest processing times, preserved the DHM bioactive components and their functionality better than HoP and other thermal treatments tested. However, although heat–sensitive compounds such as BSSL and LTF were better retained with this method, significant reductions were observed when compared to untreated samples. The DHM bacteriostatic capacity against *E. coli* was also significantly reduced after HTST and resulted in insulin degradation (**chapter 6**) and loss of the DHM procoagulant activity (**chapter 8**). Similar reductions have been reported in DHM bioactive components after HTST [16, 84, 85], but as previously discussed (**chapter 4**), large variations exist regarding the degree of degradation. In **chapter 4**, a comparison of thermal methods with the same holding time but with various heating up times revealed that longer heating up times affected the DHM functional components the most. In general, it was shown

that prolonged exposure above 55°C of DHM has a significant impact on the retention of its bioactive proteins and on their functions. Of the thermal treatments, HTST may be the optimal method, but due to the losses in the aforementioned heat–sensitive compounds, non–thermal methods may prove superior to thermal methods.

In **chapter 5**, HPP, UV–C and TUS were investigated as the most promising non–thermal alternative methods to HoP. When HPP was performed at 400 MPa, 500 MPa and 600 MPa for <3 min, the IgA, LTF, LYZ and BSSL levels were not significantly different from those of untreated samples. At the same time, these treatments resulted in comparable bacteriostatic properties and BSSL activity compared to untreated DHM. Similar findings were observed for the UV–C irradiated DHM samples, where the highest dosage tested (4863 J/L) was focused on, since it was the only dosage capable of achieving sufficient bacterial inactivation. Although TUS affected DHM's bioactive components less than HoP, significant reductions were still documented, especially on the DHM bacteriostatic properties, and BSSL level and activity. The high retention rates of DHM bioactive components documented after UV–C and HPP are in line with previous studies, as discussed in detail in **chapter 5.**

After assessing DHM safety and functionality with all combinations of treatment parameters, the most suitable ones were selected, based on the findings obtained in the current thesis and the available literature. Thus, the effect of processing on DHM insulin and procoagulant activity (**chapters 6** and **8**), was investigated with the selected optimal parameters only (HPP; 500MPa for 5 min, UV–C; 4863J/L and TUS; 6 min at 60W). Insulin levels were not affected by these treatments, and the procoagulant activity of DHM was least affected by UV–C and HPP.

In conclusion, HPP and UV–C are the best alternatives to HoP, since both methods ensure microbiological safety with only minimal effects on the DHM bioactive components. The blood clotting time after UV–C was significantly lower than after HPP ($p<0.05$), indicating that UV–C may preserve this function better (**Figure 1**). This difference could be explained by the fact that pressure may lead to damage to particles, such as EVs, to a certain degree [86].

Another possible implication of processing that should be taken into consideration, is the alteration of DHM's volatile compounds. These changes are undesired since they may affect the DHM quality and lead to off–flavors [87]. However, since DHM is provided via a nasogastric tube to VLBW infants, off–flavor is not an issue, but it might be if provided to more mature preterm infants and term neonates. One study documented significant changes in aldehydes, furans and pyrans after HoP, while HPP at 400MPa or 600MPa for 3 min, did not increase the formation of these compounds [88]. However, more intense HPP treatments (600MPa for 6 min) significantly increased such compounds [88, 89]. For UV–C, some studies concluded that it can sufficiently inactivate microbial contaminants while maintaining the milk's organoleptic characteristics, but others reported the generation of lipid peroxidation secondary products [27, 28, 90–92]. These differences may be explained by the different treatment intensities used as well as the different lipid oxidation markers measured. The alteration of DHM's volatile compounds after UV–C and HPP were not evaluated in the current thesis, but no off–flavor was detected in the treated samples, as well as no butter or butter oil formation.

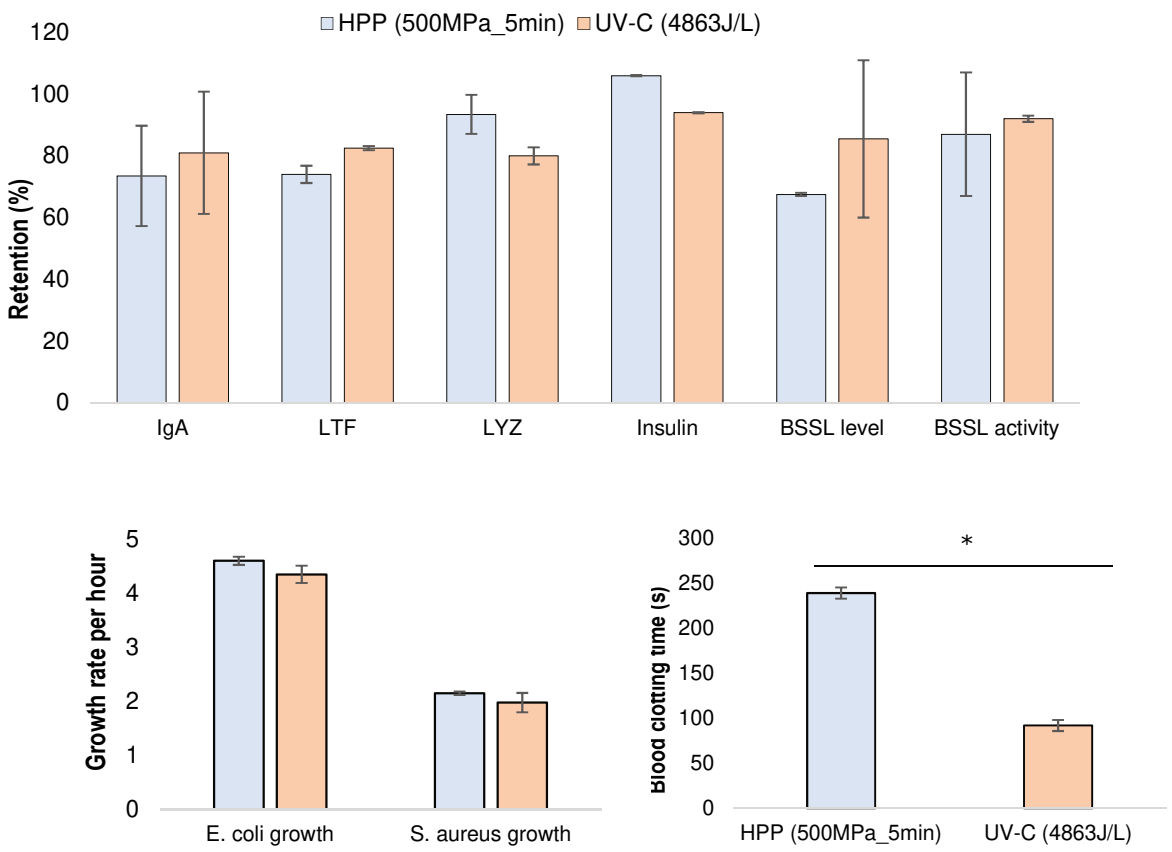


Figure 1. Retention of DHM components and bioactivities with the selected HPP and UV-C treatments

9.1.3. Cost–efficiency and implementation

Most HMBs have limited financial resources and the practices followed are usually quite costly [2]. Such practices may include DHM transportation, screening, processing and storage, as well as staff training and other administrative tasks [93]. Due to these implications, before selecting a novel processing method for implementation at a HMB, cost–consequence and cost–effectiveness analyses must be first performed.

HPP has two main limitations; the lack of an appropriate equipment for a HMB setting and the costs related to the investment and operation of the unit [2]. The estimated annual HPP cost for a HMB is approximately 7 times higher than the cost of HoP and according to HPP manufacturers, the unit must be kept in a separate room which is compliant with the conditions required for a safe operation (e.g. wall thickness, load–bearing capacity) [94]. At the moment, no HPP devices are commercially available with a size and weight suitable for a HMB environment. Although a prototype of a scaled–down HPP device weighing almost 200 kg less than common HPP units has been described, its construction and validation has not yet been performed [2].

The lack of appropriate equipment is also a limitation for the implementation of UV–C in a HMB context. However, when compared to HPP, substantially more progress has been made; a UV–C apparatus for HM processing was set up by an Australian research group (**Figure 2a,** [95]), which is the set–up used in most studies [27, 28, 35, 63, 76], while two other

devices have been additionally tested (**Figure 2b** and **2c**, [2, 91, 96]). The devices developed aimed to overcome the limited penetration depth of UV–C by stirring the milk with magnetic stirrer bars, in order to ensure low velocity flows for a homogeneous treatment. The equipment used in the current thesis was based on the set–up described in **Figure 2a**.UV–C has the advantage of being more energy–efficient than thermal pasteurization [97, 98]. In the current thesis, the overall cost of the equipment used (UV–C lamp, beaker, magnetic stirrer and stirrer base) was approximately 410, € while the ease of installation and use of this experimental set–up was a great advantage over the other methods tested. The low cost of this method is of crucial importance, as it makes the implementation of this method possible in developing countries as well.

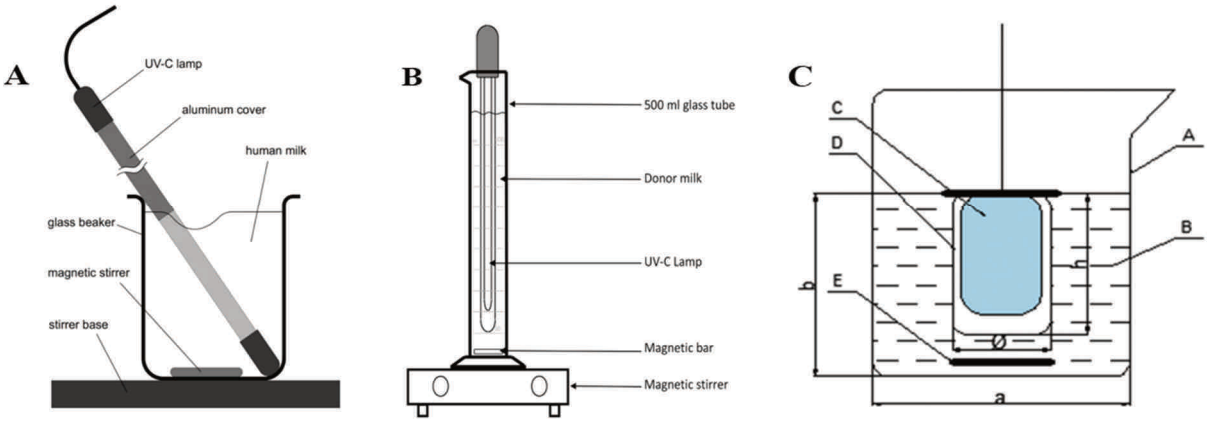


Figure 2. Schematic representation of UV–C devices used for DHM treatment. **A**; adapted from Christen et al., 2013 [27], **B**; adapted from Moro et al., 2019 [2], and **C**; adapted from Martysiak–Żurowska et al., 2017 (A: beaker, B: milk; C: filament; D: external quartz shield of filament E: magnetic stirrer, [91]).

Nevertheless, since both the power of the UV–C lamps used and the DHM turbidity affect the UV–C penetration depth, each unique UV–C device must be thoroughly tested and evaluated before enabling its usage in HMBs. The DHM volume to UV–C lamp surface area ratio may also influence the efficacy of the treatment and should be considered when such devices are tested. Apart from those issues, Christen and his colleagues showed that increased total solid concentrations in DHM samples required increased decimal reduction dosages, as this limited the penetration depth of UV–C photons [27]. However, this issue can be easily solved either by measuring their solids concentration before treatment and adjust the dosage accordingly, or by always assuming high total solid concentrations and applying a sufficiently high UV–C dosage [27]. The latter was performed in this thesis.

Next to the practical issues regarding implementation, also clinical data on its potential benefits are currently lacking. A study using preterm pigs as a preterm infant model showed that UV–C treated milk improved growth, intestinal health and systemic bacterial resistance, when compared to HoP, but additional *in vitro* and especially *in vivo* studies are needed [28].

9.2. Conclusions, future research and recommendations

The current thesis has clearly demonstrated that HPP and UV–C are superior to HoP. UV–C has the advantage of being a low–cost and easily implementable method, and as a next step, clinically significant endpoints should be evaluated, whereas the standardization of the

equipment should be a priority. The replacement of HoP with UV–C cannot be feasible before these steps are completed, and from a regulatory perspective, this process might be time–consuming. For the time being, one practical solution could be to improve the processing practices followed in HMBs, by ensuring shorter heating up and cooling down phases, in an effort to minimize the effects of thermal treatments. **Table 6** represents an overview of the advantages and disadvantages of HoP, HPP and UV–C, as well as the next steps that can be performed towards the optimization and/or the implementation of those methods.

Table 6. Current and novel processing methods; Advantages, disadvantages and future research.

Methods	Advantages	Disadvantages	Next steps
HoP	▪ Recommended method, already implemented, ease of operation ▪ Complete elimination of microbial contaminants ▪ Important nutritional components and a few functional components are not affected	▪ Prolonged exposure of DHM above critical temperatures for the preservation of its components ▪ Negatively affects the DHM bioactive proteins, completely inactivates lipases ▪ Ineffective against *B. cereus* ▪ Limited inactivation of hepatitis viruses and herpes simplex virus–1 ▪ Pasteurizer requalification is needed on a regular basis	▪ Improving the current processing times by optimizing processing parameters
HPP	▪ No heat–induced stress ▪ DHM bioactive components are better retained than after HoP, HTST and TUS ▪ Similar microbial inactivation to HoP ▪ Inactivation bacterial spores	▪ Large variations in the processing parameters tested among the available studies ▪ No device is commercially available ▪ Scaling down of the equipment is a great challenge ▪ The investment and operating costs would be much higher than HoP	▪ Testing in a HMB setting with standardized devices and processing conditions ▪ Viral inactivation, bacterial sublethal injuries, digestive kinetics, pilot studies and *in vivo* benefits should be further studied under standardized conditions
UV–C	▪ No heat–induced stress ▪ DHM bioactive components are better retained than after HoP, HTST and TUS ▪ Similar microbial inactivation to HoP ▪ Inactivation bacterial spores ▪ Low–cost and easily implementable ▪ Prototype devices exist	▪ Limited studies are available ▪ The limited penetration of the UV–C photons in opaque liquids ▪ No device is commercially available	▪ Testing in a HMB setting with standardized devices and processing conditions ▪ Viral inactivation, bacterial sublethal injuries, digestive kinetics, pilot studies and *in vivo* benefits should be further studied under standardized conditions

9.2.1. Processing optimization in HMBs

The results of this thesis showed that the current process can be further improved, and such improvements could result in higher DHM quality. As it is crucial to keep the exposure of DHM to temperatures above 55°C as limited as possible, each pasteurizer should undergo strict quality controls on a regular basis while the temperature of the heating medium and of the bottles should be well–monitored (e.g., with sensors, several temperature probes). That way, the temperature and the duration of the pasteurization cycle can be better controlled. In addition to that, keeping the DHM volume included in a pasteurization cycle the same in each bottle and at the same starting temperature, may further assist in optimizing the heating up and rapid cooling down phases. Longer heating up times are usually the result of small heat exchanging surface areas and small temperature differences between the heating medium and the milk. For that reason, the heat exchange surface to milk volume ratio and the heat transfer rate of the bottle, which depends on the material and the wall thickness, should be also considered. Updated milk banking guidelines that would include these parameters in detail may facilitate processing optimization.

Pooling DHM from a limited number of donors might solve the issue of pasteurizing different milk volumes, and it could also assist in reducing pre–pasteurization storage times. Although individualized fortification is performed nowadays, pooling may also enable the compensation for nutritional differences among the different donors [99]. However, this practice should be performed with extra caution, as there is always the risk of microbial contamination and the lack of donor traceability. Detailed milk banking guidelines on these crucial processing parameters should become available.

9.2.2. Non–thermal alternatives: future next steps

From the methods tested in this thesis, UV–C and HPP were the most promising, but the high costs of HPP and the lack of suitable equipment, prevents the implementation of this method in HMBs at the moment. UV–C is a more feasible alternative, which retains the DHM bioactive components and their functionalities ≥80%, as shown in the current thesis. The clinical significance of these findings should be next focused on. Studies comparing the effect of UV–C and HoP–treated DHM on lipid absorption and growth of preterm infants, as well as on clinical endpoints such as the incidence of late onset sepsis and necrotizing enterocolitis, should be conducted. More detailed information on the effects of this method on the physicochemical properties of DHM should also become available, and future studies may additionally include vitamins, cytokines, leukocytes and stem cells in their evaluation. In addition, UV–C was found capable of achieving a 5–$\log_{10}$ reduction of clinically relevant microbial contaminants, but as a following step, sublethal injury rates should be investigated to a greater extend. Both individual strains and multiple–strain mixtures, in vegetative forms and spores should be tested. In addition, the inactivation of viruses with clinical importance to DHM such as CMV, HIV and HTLV after UV–C should be a priority in future studies. However, before investigating all the aforementioned factors, the processing parameters and equipment should be first calibrated and adjusted for the volumes usually treated in HMBs.

9.2.3. DHM therapeutic applications

Antiviral drugs are currently available for several diseases, but issues such as the increasing emergence of resistance, side effects, and restricted therapeutic efficacy often arise. Besides that, the lack of approved medicines for certain viral diseases is also another concern [100]. HM's function as a defense mechanism against invading pathogenic microorganisms is well–known [100]. In fact, in the current thesis, DHM was found to contain abundant IgA antibodies against SARS–CoV–2, with neutralization capacity against a SARS–CoV–2 pseudovirus. This neutralization capacity was additionally observed in DHM samples of non–infected mothers, which might be attributed to the presence of other HM antiviral compounds, such as LTF and other glycoproteins, HMOs, PUFAs, EVs, and cytokines. In contrast to antiviral drugs, DHM is a safe product, of excellent tolerability and no detrimental side effects, with preventive and therapeutic potential against a broad spectrum of viruses. At the same time, this thesis showed that non–thermal methods preserve antiviral proteins such as IgA and LTF as well as the neutralization capacity against a SARS–CoV–2 pseudovirus better than HoP, thus underlying the potential of using DHM–treated with non–thermal methods in therapeutic applications. Therefore, as a next step, further –omic screens and clinical trials could be conducted to provide a better understanding of the combinatorial DHM antiviral mechanisms, while more efforts should be undertaken to unlock the therapeutic potential of DHM.

All in all, this thesis demonstrated that UV–C is the most suitable alternative to HoP. This method is more easily implementable when compared to HPP, while at the same time it provides a higher quality of DHM than HoP, and a microbiological safe end–product. As these findings suggest that UV–C may improve the health outcomes in preterm infants at which DHM is provided, continuous progress towards the standardization of the equipment is extremely important. The outcomes of this new standardized method should be then confirmed by analytics and well–designed clinical studies

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Appendices

English Summary

Human milk is a unique and dynamic biofluid, essential for the optimal growth and development of an infant. Provision of human milk to high–risk infants is of crucial importance, since it leads to a significant reduction of a number of life–threatening diseases, such as necrotizing enterocolitis and late onset sepsis. Mother's own milk is always the first feeding choice, but in case of unavailability, donor human milk represents the best alternative. Human milk banks subject donor human milk to holder pasteurization in order to ensure its safety and although most nutritional components remain unaffected by this treatment, many bioactive components suffer a significant decrease. For that reason, this thesis aimed to investigate the impact of thermal and non–thermal methods as alternatives to holder pasteurization, on donor human milk safety and functionality. First, in **chapter 2**, the analytical techniques that could be used for such analyses were described. To allow for conclusions on the suitability of a method for donor human milk processing, a workflow for donor human milk analysis was proposed, with a safety panel as the primary analytical step, followed by the assessment of the preservation of its nutritional value and functionality. The large variation in certain milk banking practices is discussed in detail in **chapter 3**, specifically in relation to pasteurization practices, such as the variation in heating up and cooling down times, but also with respect to storage and milk screening, both pre– and post–pasteurization. When the thermal treatment was optimized, as described in **chapter 4**, the immunoglobulin A and lysozyme levels and functionality after high–temperature–short–time, a method with shorter processing times than HoP, were comparable to that of untreated donor human milk. However, heat sensitive proteins such as lactoferrin and bile salt–stimulated lipase were significantly affected by all thermal treatments. In **chapter 5**, it was shown that high–pressure processing, ultraviolet–C irradiation and thermoultrasonication, preserved the donor human milk immunoglobulin A, lactoferrin, lysozyme and bile salt–stimulated lipase better than holder pasteurization, with less intense high–pressure processing and ultraviolet–C irradiation treatments preserving these proteins in levels and activities comparable to untreated donor human milk. These methods were also found to preserve the insulin concentration in donor human milk better than HoP, as shown in **chapter 6,** which is a key component for the infant's gastrointestinal development. High-pressure processing additionally preserved the milk's neutralizing capacity against SARS–CoV–2, as presented in **chapter 7**, a function eliminated with holder pasteurization. Similarly, the findings described in **chapter 8** indicate that human milk's procoagulant activity was completely destroyed by thermal treatments and among the non–thermal treatments tested, ultraviolet–C preserves this function better. In summary, this thesis suggests that optimizing thermal treatment by reducing the processing times may preserve the donor human milk bioactivity better than holder pasteurization. However, non–thermal methods such as high–pressure processing and ultraviolet–C irradiation performed even better than all thermal treatments in preserving these bioactive components, while at the same time ensuring donor human milk safety, and thus may be regarded as the most promising alternatives to holder pasteurization.

Nederlandse samenvatting

Moedermelk is een unieke en dynamische vloeistof, die essentieel is voor de optimale groei en ontwikkeling van een pasgeborene. Het verstrekken van moedermelk aan zuigelingen met een hoog ziekterisico is van cruciaal belang, aangezien het leidt tot een significante vermindering van een aantal levensbedreigende ziekten, zoals as necrotizing enterocolitis en late onset sepsis. Melk van de eigen moeder is altijd de eerste keuze, maar indien niet beschikbaar is donormoedermelk het beste alternatief. Moedermelkbanken behandelen donormoedermelk aan batch pasteurisatie om de veiligheid ervan te garanderen en hoewel de meeste voedingscomponenten niet worden beïnvloed door deze behandeling, nemen veel bioactieve componenten significant af. Om die reden was het huidige proefschrift gericht op het onderzoeken van de impact van thermische en niet–thermische behandelmethoden als alternatieven voor batch pasteurisatie, op de veiligheid en functionaliteit van donormoedermelk. Eerst worden in **hoofdstuk 2** de analytische technieken beschreven die voor dergelijke analyses kunnen worden gebruikt. Om conclusies te kunnen trekken over de geschiktheid van een behandelmethode voor donormoedermelk–, werd een workflow voor donormoedermelk –analyse voorgesteld, met een veiligheidspanel als eerste stap, gevolgd door de beoordeling van het behoud van de voedingswaarde en functionaliteit. De grote variatie in bepaalde melkbankpraktijken wordt uitgebreid besproken in **hoofdstuk 3**, specifiek in relatie tot –de uitvoering van batch pasteurisatie, zoals de variatie in opwarm– en afkoeltijden, maar ook met betrekking tot bewaring en melkscreening, zowel voor als na – batch pasteurisatie. Nadat de thermische behandeling was geoptimaliseerd zoals beschreven in **hoofdstuk 4**, waren de immunoglobuline A en lysozym niveaus en functionaliteit na ––hoge temperatuur/korte tijd, een methode met kortere verhittingsduur dan batch pasteurisatie, vergelijkbaar met die van onbehandelde donormoedermelk. Hittegevoelige eiwitten zoals lactoferrine en bile salt–stimulated lipase werden echter significant beïnvloed door alle thermische behandelingen. In **hoofdstuk 5** werd aangetoond dat–hogedrukbehandeling, ultraviolet–C doorstraling en thermo–ultrasonificatie, de donormoedermelk immunoglobuline A, lactoferrine, lysozym en bile salt–stimulated lipase beter behouden dan met batch pasteurisatie. Met minder intense hogedrukbehandeling en ultraviolet–C doorstraling worden deze eiwitten behouden op niveaus en met activiteiten die vergelijkbaar zijn met onbehandeld donormoedermelk. Deze methoden bleken ook de insulineconcentratie in donormoedermelk beter te behouden dan batch pasteurisatie, zoals wordt aangetoond in **hoofdstuk 6**, wat een sleutelcomponent is voor de gastro–intestinale ontwikkeling van de zuigeling. Hogedrukbehandeling behield bovendien het neutraliserende vermogen van de melk tegen SARS–CoV–2, zoals beschreven in **hoofdstuk 7**, een functie die wordt geëlimineerd met batch pasteurisatie. Daarnaast laten de resulten beschreven in **hoofdstuk 8** zien dat de procoagulerende activiteit van moedermelk volledig werd vernietigd door thermische behandelingen en van de geteste niet–thermische behandelingen, behoudt Ultraviolet–C deze functie het best. Samenvattend laat dit proefschrift zien dat het optimaliseren van thermische behandeling door het verkorten van de verhittingsduur, de –bioactiviteit van donormoedermelk beter kan behouden dan batch pasteurisatie. Niet–thermische methoden zoals hogedrukbehandeling en ultraviolet-C presteerden echter beter dan alle thermische behandelingen voor wat betreft het behoud van deze bioactieve componenten, terwijl ze tegelijkertijd de –veiligheid van donormoedermelk waarborgen, en kunnen daarom worden beschouwd als de meest veelbelovende alternatieven voor batch pasteurisatie.

PhD Portfolio

PhD student: Eva Kontopodi

PhD period: 2017-2021

Names of PhD supervisor(s) & co-supervisor(s):
R.M. van Elburg, J.B. van Goudoever, Kasper Hettinga, Bernd Stahl

1. PhD training

	Year	ECTS
General courses		
The AMC World of Science	2018	0.7
Specific courses		
Mass Spectrometry, Proteomics and Protein Research	2018	2.1
EndNote	2018	0.1
Introduction to R, Wageningen University & Research	2018	0.7
Seminars, workshops and master classes		
Master Class Dairy Protein Biochemistry, Wageningen University & Research	2018	1.0
VLAG Online lecture series, Wageningen University & Research	2020	0.5
Seminar on dairy chemistry by prof. Monika Pischetsrieder, Wageningen University & Research	2018	0.1
Seminar "Milk and health from an epidemiological perspective", Wageningen University & Research	2018	0.1
Wageningen Food Science Symposium, Wageningen University & Research	2020	0.2
Symposium "Food, microbiome and immunity", Wageningen University & Research	2019	0.2
Presentations		
Amsterdam Kinder Symposium-**oral presentation** *(Alternative methods of pasteurization for donor human milk preservation)*	2018	0.25
Amsterdam Kinder Symposium -**oral presentation** *(Survey on the operation of European human milk banks: "Donor milk banking: Improving the future")*	2020	0.25
Amsterdam Kinder Symposium-**oral presentation** *(The effect of different time-temperature profiles on bioactive proteins during pasteurization of donor human milk)*	2020	0.25
(Inter)national conferences		
1st International Donor Milk Research Congress, European Milk Banking Association (EMBA)- **oral presentation** *(Bacteriostatic properties and lipase activity of human milk following different processing methods)*	2018	0.5
16th International Symposium on Milk Genomics and Human Health-***poster presentation*** *(The effect of different time-temperature profiles on bioactive proteins during pasteurization of donor human milk)*	2019	0.7

5th International EMBA Congress-**oral presentation** *(The effect of different time-temperature profiles on bioactive proteins during pasteurization of donor human milk)*	2019	0.7
2nd International Donor Milk Research Congress, EMBA-**oral presentation** (*Breastmilk; a source of SARS-CoV 2 specific IgA antibodies)*	2020	0.25
18th International Symposium on Milk Genomics and Human Health-**oral presentation** *(Human Milk from Previously COVID-19-Infected Mothers: The Effect of Pasteurization on Specific Antibodies and Neutralization Capacity)*	2021	0.7
6th International EMBA Congress-**oral presentation** *("Donor milk banking: Improving the future": A survey on the operation of the European donor human milk banks)*	2021	0.7
Other		
Fundamental transition of our Food System; looking for solutions for the future of food, Symposium Nicolas Appert	2017	0.1
PhD Seminar, Wageningen University & Research	2017	0.2
Diary proteins meeting series (journal club), Wageningen University & Research	2018-2019	0.5
Monthly dairy PhD (DST) meetings, Wageningen University & Research	2017-2021	2.0

2. Teaching

	Year	**ECTS**
Tutoring, Mentoring		
Food packaging and design, Wageningen University & Research	2017-2018 (period 4)	4
Case studies on product quality, Wageningen University & Research	2017-2018 (period 4)	4
Supervising		
Medical student, BSc thesis (6 months)	2017-2018	4
Dairy science student, MSc thesis, Wageningen University & Research (6 months)	2017-2018	4
Dairy science student, MSc thesis, Wageningen University & Research (6 months)	2018-2019	4
Dairy science student, MSc thesis, Wageningen University & Research (6 months)	2018-2019	4
Dairy science student, MSc thesis, Wageningen University & Research (6 months)	2018-2019	4
Dairy science student, MSc thesis, Wageningen University & Research (6 months)	2019-2020	4
Dairy science student, MSc thesis, Wageningen University & Research (6 months)	2019-2020	4
Food microbiology student, MSc thesis, Wageningen University & Research (6 months)	2019-2020	4
Food microbiology student, MSc thesis, Wageningen University & Research (6 months)	2018-2019	4
Other		
Thesis ring in food microbiology supervisor (10h in total), Wageningen University & Research	2018-2020	0.35

3. Parameters of Esteem

Awards and Prizes	**Year**
Student travel award for oral presentation, International Symposium on Milk Genomics and Human Health	2021

4. Publications

Peer reviewed (This thesis)	**Year**
van Keulen, B. J., Romijn, M., Bondt, A., Dingess, K. A., Kontopodi, E., van der Straten, K., ... & van Goudoever, J. B. (2021). Human milk from previously covid-19-infected mothers: The effect of pasteurization on specific antibodies and neutralization capacity. **Nutrients**, 13(5), 1645.	2021
Kontopodi, E., Arslanoglu, S., Bernatowicz-Lojko, U., Bertino, E., Bettinelli, M.E., Buffin, R., Cassidy, T., van Elburg, R.M., Gebauer, C., Grovslien, A. and Hettinga, K. (2021). "Donor milk banking: Improving the future". A survey on the operation of the European donor human milk banks. **Plos one**, 16(8), p.e0256435	2021
Mank, E. Kontopodi, A.C. Heijboeret al., van Elburg, R.M., Hettinga, K., van Goudoever, J.B. and van Toledo, L (2021). Thermoultrasonication, ultraviolet-C irradiation, and high-pressure processing: Novel techniques to preserve insulin in donor human milk. **Clinical Nutrition** 40(11), pp.5655-5658	2021
Kontopodi, E., Hettinga, K., Stahl, B., van Goudoever, J.B. and Van Elburg, R., (2021). Testing the Effects of Processing on Donor Human Milk: Analytical Methods. **Food Chemistry**, p.131413.	2021
Kontopodi E, Boeren S, Stahl B, Goudoever JB Van. High-Temperature Short-Time Preserves Human Milk ' s Bioactive Proteins and Their Function Better Than Pasteurization Techniques With Long Processing Times. **Front Pediatr**. 2022;9,1–12.	2022
Yong Hu, Eva Kontopodi, Elise Mank, Chris H.P. van den Akker, Johannes B. van Goudoever, Kasper Hettinga, Ruurd M. van Elburg, Johannes Thaler, and Rienk Nieuwland (2022*). Processing methods of donor human milk evaluated by a blood plasma clotting assay*. **Innovative Food Science & Emerging Technologies**, 102938, 1466-8564	2022
Peer reviewed (Other)	**Year**
Liu, Y., Xiong, L., Kontopodi, E., Boeren, S., Zhang, L., Zhou, P., & Hettinga, K. (2020). Changes in the milk serum proteome after thermal and non-thermal treatment. **Innovative Food Science & Emerging Technologies**, 66, 102544.	2020
Courraud, J., Quist, J. S., Kontopodi, E., Jensen, M. B., Bjerrum, P. J., Helge, J. W., & Sørensen, K. (2020). *Dietary habits, metabolic health and vitamin D status in Greenlandic children*. **Public health nutrition,** 23(5), 904-913	2020

Contributing authors

Authors	Contribution	Chapters
Johannes B. van Goudoever	Conceptualization, Supervision, Investigation, Resources, Reviewing and Editing.	1-9
Ruurd M. van Elburg	Conceptualization, Supervision Investigation, Resources, Reviewing and Editing.	1-9
Kasper Hettinga	Conceptualization, Supervision, Investigation, Resources, Reviewing and Editing.	1-9
Bernd Stahl	Conceptualization, Supervision, Investigation, Resources, Reviewing and Editing.	1-9
Sertac Arslanoglu	Conceptualization, Investigation.	3
Enrico Bertino	Conceptualization, Supervision, Investigation, Reviewing and Editing.	3
Rachel Buffin	Conceptualization, Investigation.	3
Tanya Cassidy	Investigation, , Reviewing and Editing.	3
Guido E. Moro	Conceptualization, Supervision, Investigation, Reviewing and Editing.	3
Jean-Charles Picaud	Conceptualization, Investigation.	3
Gillian Weaver	Conceptualization, Investigation, Reviewing and Editing.	3
Urszula Bernatowicz-Lojko	Investigation.	3
Maria Enrica Bettinelli	Investigation.	3
Corina Gebauer	Investigation.	3
Ioanna Ioannou	Investigation.	3
Anne Grovslien	Investigation.	3
Daniel Klotz	Investigation, Reviewing and Editing.	3
Radmila Mileusnić-Milenović	Investigation.	3
Aleksandra Wesolowska	Conceptualization, Supervision, Investigation, Project administration, Reviewing and Editing.	3
Sjef Boeren	Methodology, Validation, Writing-Reviewing and Editing	4, 5
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Albert Bondt	Conceptualization, Software, Validation, Formal analysis, Investigation, Data curation, Reviewing and Editing.	7

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Albert J. R. Heck	Conceptualization, Supervision, Software, Validation, Formal analysis, Investigation, Data curation, Reviewing and Editing.	7
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Chris H.P. van den Akker	Methodology, Supervision, Formal Analysis, Investigation, Reviewing and Editing.	8
Johannes Thaler	Conceptualization, Supervision, Methodology, Validation, Formal Analysis, Investigation, Reviewing and Editing.	8
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