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Review

Testing the effects of processing on donor human Milk: Analytical methods

Eva Kontopodi [a,b,*], Kasper Hettinga [b], Bernd Stahl [c,d], Johannes B. van Goudoever [a], Ruurd M. van Elburg [a]

[a] Amsterdam UMC, University of Amsterdam, Vrije Universiteit, Emma Children's Hospital, Human Milk Bank, Amsterdam, the Netherlands
[b] Food Quality and Design Group, Wageningen University & Research, the Netherlands
[c] Department of Chemical Biology & Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands
[d] Danone Nutricia Research, Utrecht, the Netherlands

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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.

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 (Demarchis, Israel-Ballard, Mansen, & Engmann, 2017; WHO, 2001).

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 min followed by rapidly cooling it down to < 10 °C (Arslanoglu et al., 2010; Moro et al., 2019). 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 (Peila et al., 2016). 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 (Peila et al., 2017).

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 (Peila et al., 2017). 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 (Klotz et al., 2017; Peila et al., 2017; 2016). As a

* Corresponding author at: Emma Children's Hospital, Amsterdam UMC, Meibergdreef 9, 1000 DE Amsterdam, the Netherlands.
E-mail address: e.kontopodi@amsterdamumc.nl (E. Kontopodi).

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

DHM safety analytical panel	Methodological approach	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 contamination levels should be selected for inoculation with the appropriate bacterial strains
	Culture-independent techniques	Molecular-based applications *(PCR, genomic testing, MALDI-TOF MS)*		
Bacteria inactivation	Challenge tests	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, Enterococci, Streptococci, Enterobacteriaceae, Pseudomonas, Bacilli*). 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
Virus inactivation	Seropositive mothers	DNA extraction and PCR amplification, antibody detection assays and MS methods, neutralization assays	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
	Challenge tests	PRA, TCID50, early antigen IF, cell culture toxicity, GFP indicator cells, RNA assay, RT activity, SEAP reporter, PBMC neutralization assay		

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. 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 (Martín et al., 2004). 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 (Committee on Nutrition; Section on Breastfeeding; Committee on Fetus and Newborn, 2017; Escuder-Vieco et al., 2018).

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 (Arslanoglu et al., 2010; Weaver et al., 2019). 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) (Clarke & May, 2000; Pitino, O'Connor, & McGeer, 2021). To date, transmission of SARS-CoV-2 through human milk has not been proven (Kumar, Meena, Yadav, & Kumar, 2021).

2.1. 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 (Arslanoglu et al., 2010; Pitino et al., 2021). 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 (Weaver et al., 2019). Most human milk banking guidelines recommend discarding DHM with total bacterial counts $> 10^5$ colony forming units (CFU)/mL (Institute (NICE), 2010; Arslanoglu et al., 2010; Weaver et al., 2019). 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 (Kontopodi et al., 2021; Mallardi et al., 2021). Although cases of *B. cereus* infection in premature infants are extremely rare, neonatal sepsis due to *B. cereus* can be fatal (Cormontagne et al., 2021). 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 (Institute (NICE), 2010). In addition, milk banking guidelines indicate that post-processing, all pathogens should be absent (Institute (NICE), 2010; Arslanoglu et al., 2010; Weaver et al., 2019).

2.2. Safety: Analytical panel

As the recipients of DHM are high-risk infants, all processing methods must meet the highest safety standards (Peila et al., 2017). 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.

2.2.1. 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 (Adzitey, Huda, & Ali, 2013). However, these methods are laborious, time consuming, highly dependent on the skills of the person performing the analysis and the equipment used (Váradi et al., 2017). 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) (Almutawif, Hartmann, Lloyd, Erber, & Geddes, 2017). Bacterial species can be further identified with colony morphology, Gram staining, and standard biochemical tests (Váradi et al., 2017). 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 (Landers & Updegrove, 2010; Sauget, Valot, & Bertrand, 2017). These molecular-diagnostic techniques were initially developed to circumvent the limitations of the standard laboratory phenotyping methods (Bou, Fernández-Olmos, García, Sáez-Nieto, & Valdezate, 2011). 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 (Váradi et al., 2017). Distinction among species with comparable phenotypic characteristics is possible with those methods, as well as differentiation among strains of the same species (Yarza et al., 2014). 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 (Adzitey et al., 2013; Franco-Duarte et al., 2019; Váradi et al., 2017). For further discrimination between viable and dead microbial cells, a selective membrane impermeable dye such as propidium monoazide is commonly used (Humbert, 2019).

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.

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*) (Arslanoglu et al., 2010; Christen et al., 2013b; Landers & Updegrove, 2010; Marín, Martín, Mediano, Del Campo, & Rodríguez, 2016; Martín et al., 2004; Patel et al., 2017). 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 (Rocha-Pimienta, Martillanes, Ramírez, Garcia-Parra, & Delgado-Adamez, 2020). Finally, the inactivation achieved is compared to the minimum required inactivation level (Christen et al., 2013b).

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 (Donalisio et al., 2018; Hamprecht et al., 2004; Lloyd et al., 2016; Pitino et al., 2021; Unger et al., 2020). Another study included DHM from hepatitis B seropositive mothers that were submitted to DNA extraction and were further assessed with PCR assays (de Oliveira et al., 2009). Assessment of HIV viral infectivity before and after DHM heating using a peripheral blood mononuclear cell neutralization assay has also been reported (Inactivation, 2010).

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*) (Leite, Migotto, & Virginia, 2019). In HM, ALP may also serve as a beneficial enzyme (Yang et al., 2019). The activity of this enzyme is usually assessed spectrophotometrically with the addition of p-nitrophenyl phosphate as a substrate, by calorimetric or fluorometric assays (Christen et al., 2013b; Escuder-Vieco et al., 2018; Leite et al., 2019; Yang et al., 2019). 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 (Institute (NICE), 2010). In addition, when DHM was subjected to UV-C, although sufficient microbiological inactivation was achieved, no loss of activity was documented (Christen et al., 2013a). 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.

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 (Ballard & Morrow, 2013; Monaco & Kim, 2015; Thurl, Munzert, Boehm, Matthews, & Stahl, 2017). Due to the differences in lactational stage, maternal and environmental factors, the nutrient composition among different HM samples can be quite variable (Ballard & Morrow, 2013; Monaco & Kim, 2015). 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.

3.1. Macronutrient and micronutrient content

HM proteins can be classified into three major groups: caseins, whey 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 (Andreas, Kampmann, & Mehring Le-Doare, 2015; Donovan, 2019). The whey protein fraction includes many proteins, with α-lactalbumin and lactoferrin among the most abundant. The whey/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

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	Rapid spectroscopic methods Reference methods	Measurements of energy, fat, protein, lactose, oligosaccharides, and total solids using MIR, FT-IR or NIR spectroscopy 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, enzymatic assays with colorimetric detection, HPLC, LC-MS) 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)	Especially with regards to the estimation of the DHM protein and lipid content, significant differences have been reported between the rapid spectroscopic methods and the reference laboratory methods Reference laboratory 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

the total nitrogen in mature HM (Ballard & Morrow, 2013). Nutritionally, HM proteins serve as amino acid sources. Since the amino acid composition of whey proteins and caseins differs, and their ratio differs throughout lactation, the HM amino acid profile varies as well (Nutritional, 2003).

HM lipids are known to be highly variable. Factors such as the lactation stage and maternal diet have been shown to influence their composition (Lyons, Ryan, Dempsey, Ross, & Stanton, 2020). HM lipids are considered as the main energy source, accounting for approximately 50% of total HM energy content (Andreas et al., 2015; Floris, Stahl, & Abrahamse-Berkeveld, 2020). Triacylglycerides (TAGs) represent 98% of the HM lipids while the remaining fraction consists of diacylglycerides, monoacylglycerides, phospholipids, cholesterol, and free fatty acids (Andreas et al., 2015). Fatty acids that are available in high concentrations in HM include linoleic, palmitic, and oleic acid (Ballard & Morrow, 2013; Siziba et al., 2020). 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) (Monaco & Kim, 2015).

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 (Nommsen, Lovelady, Heinig, Lönnerdal, & Dewey, 1991). In mature HM, lactose concentration is 67–78 g/L (Ballard & Morrow, 2013). HMOs concentration in colostrum is 20.9 g/L on average and in mature HM, 12.9 g/L (Andreas et al., 2015; Thurl et al., 2017). HMOs are considered nonnutritive to the infant, but with great functional properties (Ballard & Morrow, 2013).

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 (Monaco & Kim, 2015). Apart from vitamin D, which can be found in the whey 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 (Ballard & Morrow, 2013; Monaco & Kim, 2015). 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 (Monaco & Kim, 2015).

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 (Arslanoglu et al., 2010; Peila et al., 2016). Lactose, fatty acids, the majority of minerals and vitamins A, D, and E are included in those heat-stable factors (Arslanoglu et al., 2010; Mohd-Taufek et al., 2016). 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 (Peila et al., 2016). 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 (Christen et al., 2013b). 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.

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 (García-Lara et al., 2012; Perrin et al., 2019; Smilowitz, Gho, Mirmiran, German, & Underwood, 2014). These methods are based on the interaction between specific infrared irradiation wavelengths with various chemical groups of HM components (García-Lara et al., 2012). The technology employed by these devices allows for rapid HM analysis, using only a small amount of HM (Elsohaby et al., 2018). However, measurements of the total carbohydrate content have been reported to be less accurate compared to other nutrients, among the available devices (Perrin et al., 2019). Especially for lactose quantification, MIR spectroscopy may give poor results, as the presence of oligosaccharides can affect the measurement (Wu, Jackson, Khan, Ahuja, & Pehrsson, 2018). 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 (Peila et al., 2016). As a general guideline, the calibration, validation, and quality assurance of HMAs must be ensured, in order to obtain accurate and precise measurements (Kwan et al., 2020).

Reference laboratory analytical methods are considered as the gold standard (Groh-Wargo, Valentic, Khaira, Super, & Collin, 2016). For total protein determination, the Kjeldahl or Dumas methods are routinely used. These methods are based on total nitrogen estimation (Dupont, Croguennec, & Pochet, 2018; Groh-Wargo et al., 2016). 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 (Dupont et al., 2018; Bo Lönnerdal & Glazier, 1987). Studies comparing the protein values obtained by these methods to the values measured with Kjeldahl concluded that the BCA assay showed the greatest precision (Giuffrida et al., 2019; Keller & Neville, 1986). 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 (Bo Lönnerdal & Glazier, 1987). 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 (Cortes-Ríos, David, & Medina, 2020; Giuffrida et al., 2019).

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 (Martysiak-Żurowska et al., 2017a; Shi et al., 2011; Wu et al., 2018).

High performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is regarded as a selective and sensitive method for lactose quantification (Giuffrida et al., 2019). 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 (Comparison, 2012), even though significant differences in the DHM macronutrient concentration between HMA and the reference laboratory methods have been described (Casadio et al., 2010; Peila et al., 2016). Therefore, for the analysis of individual DHM components, reference laboratory methods may be preferred, in order to obtain the most accurate results.

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 (Hampel, Dror, & Allen, 2018). UPLC-MS/MS, specifically, is considered to have offered improved resolution and analytical sensitivity in such analyses (Wu et al., 2018). 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 (Hampel et al., 2018).

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 (Peila et al., 2016).

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 (Hampel et al., 2018). These methods have been shown to be superior to the colorimetric approaches previously used for mineral analysis (Hampel et al., 2018).

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 (Monaco & Kim, 2015). 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 (Ballard & Morrow, 2013; Peila et al., 2017; 2016). Since current evidence suggests that HMOs are not affected by HoP (Peila et al., 2016), the analytical methods available to assess these components are not further discussed in the present study.

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 whey fraction [42]. The most abundant immunoglobulins in HM are sIgA, followed by sIgG and sIgM (Monaco & Kim, 2015). 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 (Monaco & Kim, 2015). 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 (Ballard & Morrow, 2013; Monaco & Kim, 2015). 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 (Dallas et al., 2015).

LTF represents approximately 15% to 20% of the total HM protein content and is considered as one of the principal antimicrobial HM components (Lönnerdal, 2017). 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 (Lönnerdal, 2016).

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 (Monaco & Kim, 2015). When acting alone, LYZ is capable of killing gram-positive bacteria by lysing the proteoglycan matrix located in their cell wall (Lönnerdal, 2017). 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 (Nutritional, 2003; Monaco & Kim, 2015).

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
	Targeted protein determination	Detection of a targeted antigen using a specific enzyme-labelled antibody (e.g. ELISA, RIA, electrochemiluminescence immunoassays)	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
		Measurement of the ions specific to the peptides that correspond to the protein of interest through a triple quadrupole MS (LC-MRM/MS)	
		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	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)
	Functionality assays	Enzymatic activity assays, antibacterial activity assays, assays that determine the neutralization capacity of specific antibodies	
Lipidome	Untargeted and targeted 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.

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 (Fredrikzon, Hernell, Bläckberg, & Olivecrona, 1978). 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 (Andersson, Sävman, Bläckberg, & Hernell, 2007; Christen et al., 2013b).

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 (Nutritional, 2003).

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 (Andreas et al., 2015; Ballard & Morrow, 2013). These compounds are implicated in a wide range of cellular functions, such as infant growth, neurobehavioral and brain development (Andreas et al., 2015; Dei Cas et al., 2020). In most studies, HoP did not reduce the total DHM lipid content or affect the FA profile (Peila et al., 2016). However, intense non-thermal processing methods may lead to formations of lipid oxidation (Moro et al., 2019). For example, HPP at 600 MPa for durations longer than 3 min was shown to result in the formation of compounds associated with lipid oxidation, such as aldehydes (Contador, Delgado, García-Parra, Garrido, & Ramírez, 2015; Moltó-Puigmartí, Permanyer, Castellote, & López-Sabater, 2011). Similarly, volatile compounds from lipid oxidation have been detected after extended ultrasonication of bovine milk (Chouliara, Georgogianni, Kanellopoulou, & Kontominas, 2010; Juliano et al., 2014), whereas for UV-C, some studies did report the occurrence of oxidation, while others didn't (Christen et al., 2013b; Li et al., 2017; Martysiak-Żurowska et al., 2017b; Matak et al., 2007) which may depend on the treatment intensity.

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 (Peila et al., 2016). 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 (Contrepois et al., 2018; Sobsey et al., 2020). Table 3 represents an overview of the analytical methods available to evaluate the effect of a processing method on DHM functionality.

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 (Peila et al., 2016). Cytokines, hormones, and growth factors are mostly characterized by using specific ELISA or RIAs (Peila et al., 2016).

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) (Konstantinou, 2017). These substances can be detected by a colorimetric reaction following the binding of an antigen to a specific enzyme-labeled antibody (Konstantinou, 2017; Pandey, Varshney, & Sudini,

2019). 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 (Bertino et al., 1996; Hnasko, Lin, Mcgarvey, & Stanker, 2011; Pandey et al., 2019; Zhu, Garrigues, Van Den Toorn, Stahl, & Heck, 2019).

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 (Grange, Thompson, Lambert, & Mahajan, 2014). Since radiolabeled antigens are used, interference risks from the sample itself are limited (Grange et al., 2014). 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 (Bagci et al., 2017; Colazo, Ambrose, Kastelic, & Small, 2008; Grange et al., 2014).

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 (Baro et al., 2011; Lönnerdal, 2010; Altendorfer et al., 2015). 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 (Affolter, Grass, Vanrobaeys, Casado, & Kussmann, 2010; Bär et al., 2019). 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. (Bär et al., 2019). 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 (Güzel et al., 2018). Therefore, ensuring appropriate fraction preparation of complex mixtures is of particular importance (Beretov, Wasinger, Graham, Millar, & Kearsley, 2014).

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.

4.3.2. Untargeted protein profiling

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 (Zhu & Dingess, 2019). These approaches are gaining more ground recently, due to the amount of information that can be acquired (Peila et al., 2016). 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 (Liu et al., 2020; Zhang et al., 2016).

For studying the HM proteome, HM is usually fractionated into its major protein classes (caseins, whey and MFGM proteins). The majority of the bioactive proteins in HM can be found in the whey fraction, also called serum fraction, which can be isolated by ultracentrifugation of HM that has been previously skimmed (Hettinga et al., 2011). 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 (Zhang et al., 2016).

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 (Wiśniewski, 2017). Both labelled and label-free proteomics have been combined with FASP and subsequently LC-MS/MS analyses to accurately determine the DHM proteome (Hettinga et al., 2011; Zhang, van Dijk, & Hettinga, 2017).

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 (Christen et al., 2013a; Peila et al., 2016). 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 (Christen, Lai, & Hartmann, 2012; 2013b; Peila et al., 2016). 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 (Christen et al., 2012; 2013b; Peila et al., 2016). Lastly, for the detection of specific HM antibody responses, mostly ELISA assays or MS-based methods with complimentary neutralization assays are used (Asensi, 2006; van Keulen, Karlijn, van der Straten, & den Boer, 2020).

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 (Christen et al., 2013a; Silvestre Castelló et al., 2008; Van Gysel, Cossey, Fieuws, & Schuermans, 2012). 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 (Christen et al., 2013a). 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 (Peila et al., 2016).

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 (Christen et al., 2013a; Effect, 2020). 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 (Christen et al., 2013a; Effect, 2020).

4.3.4. Lipidomics

Lipidomics approaches, both targeted and untargeted, may offer a comprehensive and quantitative description of the HM lipidome profile.

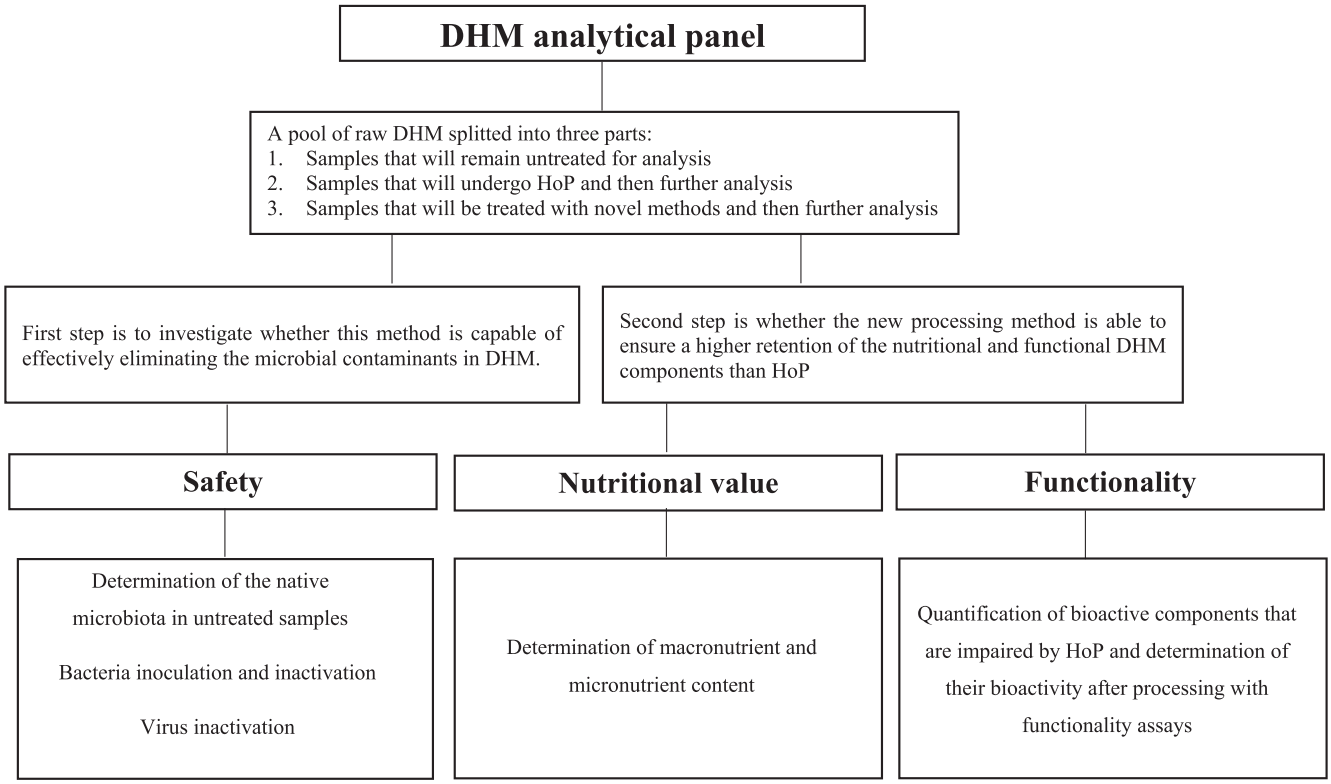

Fig. 1. Experimental workflow for validating new processing methods for DHM processing.

Such approaches are widely applied in biological research, but their application in HM is quite recent (George, Gay, Trengove, & Geddes, 2018). 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 (George et al., 2018). 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) (Lee & Yokomizo, 2018). 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) (Contrepois et al., 2018; Dingess et al., 2017). In contrast with targeted lipidomics, untargeted approaches provide a detailed analysis of the lipidome, but they are often used semi-quantitatively (Contrepois et al., 2018; George et al., 2018). 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 (George et al., 2018). The application of lipidomics in HM could be further improved with the development of a wider lipidome database and a more standardized analytical workflow (George et al., 2018).

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 (Turoli, Testolin, Zanini, & Bellù, 2004). HPLC with fluorescence detection is also commonly used for the determination of malondialdehyde, which is a lipid peroxidation end-product (Li et al., 2017). 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 (Elisia & Kitts, 2011; Li et al., 2017). Lastly, acid degree values analysis together with sensory evaluation are often used as an indicator of off-flavors in milk (Matak et al., 2007).

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 (Cacho & Lawrence, 2017). 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 (Briere, McGrath, Jensen, Matson, & Finck, 2016; Ninkina et al., 2019). 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 (Briere et al., 2016; Trend et al., 2015).

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 (Holscher, Davis, & Tappenden, 2014; Rodríguez-Camejo et al., 2020). 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 (Rodríguez-Camejo et al., 2020).

The role of HM exosomes and the microRNAs (miRNAs) they contain, on the infant's immune function and cellular development, is another emergent topic (Lönnerdal, 2019). 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 (Smyczynska et al., 2020). Several methods are currently available for exosome purification, with differential centrifugation, as well as solution sedimentation and low-speed centrifugation being most commonly

used (de la Torre Gomez, Goreham, Bech Serra, Nann, & Kussmann, 2018). 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 proteomics (de la Torre Gomez et al., 2018). To further study the miRNAs in HM, miRNA isolation, library preparation, and subsequent sequencing is usually performed (van Herwijnen, Driedonks, Basten, Theresa, & Corné, 2018; Smyczynska et al., 2020). 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 (Smyczynska et al., 2020).

Lastly, there's a large number of studies available documenting processing-induced damage to bovine milk (Abbring et al., 2020; Liu et al., 2020; Effect, 2020). 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.

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. Fig. 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.

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