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NEUTRALIZING ACTIVITY AGAINST SARS-COV-2 OF HUMAN BREAST MILK FROM COVID-19 POSITIVE AND NEGATIVE LACTATING MOTHERS

MED/38 General and Specialised Pediatrics

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Abstract (Italian)

L'alimentazione svolge un ruolo fondamentale per la salute umana, soprattutto nella popolazione pediatrica e neonatale. Il latte materno è senza dubbio l'alimento migliore per i neonati, sia per i benefici dovuti al suo contenuto nutrizionale sia per la presenza di numerose sostanze funzionali e bioattive. Le sostanze bioattive presenti nel latte materno svolgono diverse funzioni che promuovono la salute del neonato sia a breve che a lungo termine. Tra le importanti proprietà funzionali del latte umano vi è la sua spiccata attività antimicrobica e, in particolare, antivirale. La pandemia di SARS-CoV-2, l'agente patogeno alla base della COVID-19, nella sua drammaticità, è stata lo spunto per la ricerca scientifica globale, per rivalutare e testare composti con presunta o comprovata attività antivirale. La presenza di una quantità considerevole di questi composti antivirali nel latte materno spiegherebbe la sua attività antivirale non specifica nei confronti di molti virus, tra cui il SARS-CoV-2. Date queste premesse, è stato condotto uno studio per indagare l'attività antivirale non specifica del latte materno, nei confronti del SARS-CoV-2. Sono stati raccolti campioni di latte materno da madri con tampone nasofaringeo positivo per la presenza del virus (gruppo A) e da madri con tampone nasofaringeo negativo, non vaccinate contro il virus e con anamnesi negativa per l'infezione COVID-19. I campioni sono stati raccolti nei seguenti timepoints: 2 giorni dopo il parto (T0, colostro), 7 giorni dopo il parto (T1, latte di transizione) e 20 giorni dopo il parto (T2, latte maturo). I campioni raccolti sono stati poi analizzati per valutare la microneutralizzazione NT_{50} secondo il metodo di Reed e Muench. Il saggio di neutralizzazione ha analizzato l'effetto citopatico causato dalla replicazione

virale in una popolazione di cellule VERO poste in coltura, incubate con i campioni di latte materno e infettate con SARS-CoV-2. Per valutare la presenza di attività anticorpale eventualmente responsabile della neutralizzazione, i campioni di entrambi i gruppi A e B sono stati analizzati con kit ELISA per la ricerca di anticorpi diretti conto SARS-CoV-2.

I risultati hanno confermato la capacità dei campioni provenienti da madri positive (gruppo A) di neutralizzare il virus, con effetto decrescente da T0 a T2. I test ELISA su questi campioni hanno mostrato una diretta correlazione tra il titolo anticorpale presente nel campione e la capacità neutralizzante dello stesso. Per quanto riguarda il gruppo B, madri sieronegative per il virus, anche i campioni di questo gruppo hanno mostrato una attività neutralizzante, sempre decrescente nei vari timepoints, senza una diretta correlazione tra capacità neutralizzante e titolo anticorpale nel campione. La presenza di un certo titolo anticorpale anche nei campioni delle madri del gruppo B è da ascriversi alla presenza di Immunoglobuline A secretorie con cross-reattività nei confronti di SARS-CoV-2, dato già descritto in letteratura, tuttavia senza una correlazione tra titolo e capacità di neutralizzazione. I risultati di questo studio dimostrano quindi che il latte materno possiede composti, diversi dagli anticorpi, in grado di neutralizzare il virus SARS-CoV-2, con effetto più spiccato nel colostro e meno evidente nel latte maturo. Sono stati inoltre condotti alcuni test preliminari sugli stessi campioni per valutare la natura dei composti responsabili di questo effetto. I risultati preliminari hanno mostrato una diretta correlazione tra capacità neutralizzante del campione e la quantità di acido eicosatrienoico (20:3 ω-9) il cui eventuale effetto antivirale non è ancora stato descritto in letteratura. Inoltre, test pilota successivi allo studio hanno dimostrato un effetto neutralizzante crescente di concentrazioni crescenti di

oligosaccaridi del latte materno (da 0,12 mg/dL a 0,5 mg/dL), maggiormente rappresentati nel colostro che nel latte maturo.

Concludendo, il latte materno ha una capacità antivirale innata e indipendente dagli anticorpi specifici presenti. La prosecuzione della ricerca scientifica in questa direzione è necessaria per comprendere quale composto, o sinergia di composti, possa essere responsabile di questo effetto.

Abstract (English)

Nutrition plays a fundamental role in human health, even more so for the paediatric and neonatal populations. Breast milk is undoubtedly the best food for newborns, both for the benefits due to its nutritional content and for the presence of numerous functional and bioactive substances. The bioactive substances present in breast milk perform several functions that promote the short- and long-term health of the newborn. Among the important functional properties of human milk, its marked antimicrobial and, in particular, antiviral activity has a paramount role. The SARS-CoV-2 pandemic, the pathogen behind COVID-19, in its dramatic nature, was the cue for global scientific research to rediscover and test compounds with presumed, or proven, antiviral activity. The presence of a considerable amount of these antiviral compounds in breast milk would explain its non-specific antiviral activity against many viruses, including SARS-CoV-2. Given these premises, a study was conducted to investigate the non-specific antiviral activity of breast milk, thus independent of the presence of antibodies, against SARS-CoV-2. Breast milk samples were collected from mothers with a positive nasopharyngeal swab for the presence of the virus (group A) and from mothers with a negative nasopharyngeal swab, not vaccinated against the virus and with a negative history of COVID-19 infection. Samples were collected at the following timepoints: 2 days postpartum (T0, colostrum), 7 days postpartum (T1, transitional milk), and 20 days postpartum (T2, mature milk). The collected samples were then analysed to assess NT_{50} microneutralization according to the Reed and Muench method. The neutralisation assay analysed the cytopathic effect caused by viral

replication in a population of VERO cells placed in culture, incubated with breast milk samples, and infected with SARS-CoV-2. To assess the presence of antibody activity possibly responsible for the neutralisation, samples from both groups A and B were tested with ELISA kits for antibodies directed against SARS-CoV-2.

The results confirmed the ability of samples from positive mothers (group A) to neutralise the virus, with a decreasing effect from T0 to T2. ELISA tests on these samples showed a direct correlation between the antibody titre present in the sample and the neutralising capacity of the sample. As for group B, mothers seronegative for the virus, samples also showed neutralising activity, decreasing at time points, without a direct correlation between neutralising capacity and antibody titre in the sample. The presence of a certain antibody titre also in the samples of group B mothers is primarily attributable to the presence of secretory immunoglobulin A with cross-reactivity against SARS-CoV-2, a finding already described in the literature, but without a correlation between titre and neutralising capacity. The results of this study therefore demonstrate that breast milk possesses compounds, other than antibodies, capable of neutralising the SARS-CoV-2 virus, with the effect being more pronounced in colostrum and less evident in mature milk. Preliminary tests were also conducted on the same samples to assess the nature of the compounds responsible for this effect. Preliminary results showed a direct correlation between the neutralising capacity of the sample and the amount of eicosatrienoic acid (20:3 ω-9), the possible antiviral effect of which has not yet been described in the literature. In addition, pilot tests following the study showed an increasing neutralising effect of increasing concentrations of oligosaccharides in

breast milk (from 0.12 mg/dL to 0.5 mg/dL), which are more represented in colostrum than in mature milk.

In conclusion, breast milk has an innate antiviral capacity, exerted independently of the specific antibodies. Future studies are needed to understand which compound, or synergy of compounds, may be responsible for this effect.

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Chapter 1

Introduction

1.1 Human milk as a tailored, complex biological system

The close correlation between health and nutrition has been known since ancient times. In fact, a famous aphorism by Hippocrates of Cos, the father of medicine, states, 'Let food be your medicine and medicine your food'. This close correlation, abandoned for most of so-called “modern times”, has come back into the limelight in recent decades as researchers and scientists around the world rediscover the power of nutrition in preventing, and in some cases treating, most diseases that afflict humans (Bennett et al., 2015).

Indeed, it is rare to find an unhealthy condition that does not find among its causes or in its treatment a particular focus on nutrition. In the paediatric age, when systems, organs, and apparatuses are still developing, this combination of health and nutrition is even closer and more evident (K. M. Hurley et al., 2016).

The scientific literature now agrees that the first 1,000 days of a human being's life, including gestation and the intrauterine period, are a unique and fundamental time when nutrition, together with other environmental factors, plays a very important role in modulating the individual's short- and long-term health outcomes (Figure 1) (Mayneris-Perxachs & Swann, 2019). Among the many environmental factors that, thanks to what we now know to be the effect of epigenetics, are capable of influencing human health throughout life, nutrition certainly plays a predominant role (Mayneris-Perxachs & Swann, 2019).

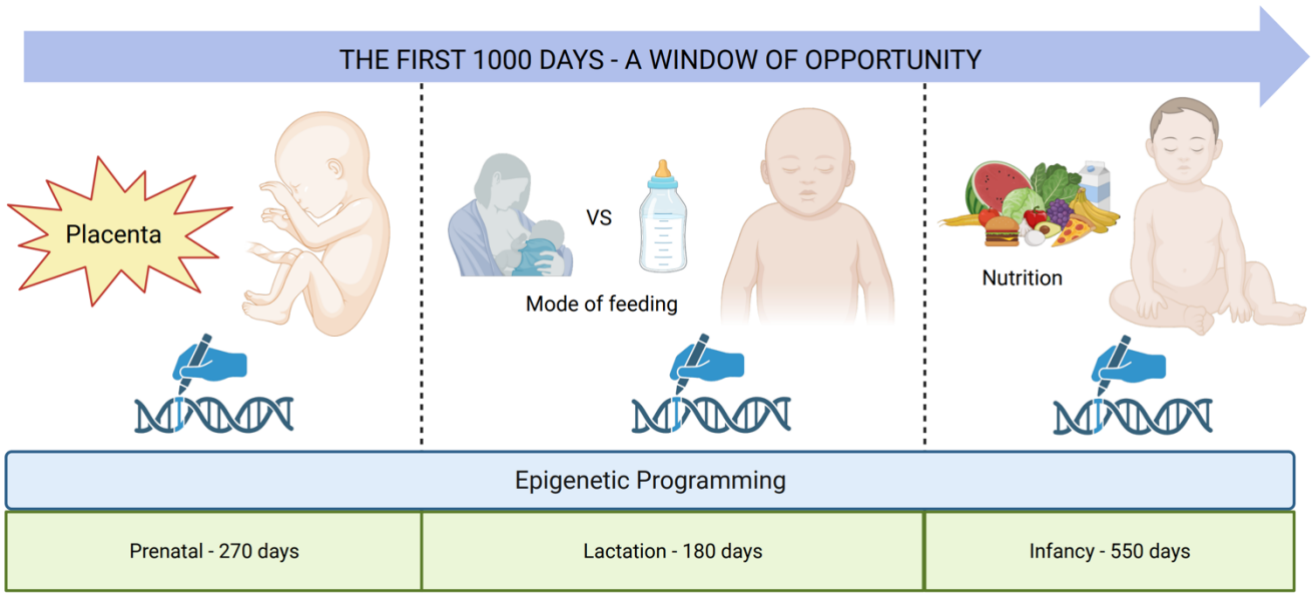

Figure 1. The epigenetic effect of nutrition during the first 1000 days of life. Created with Biorender.com

In this context, during the first year of life, and even more so during the neonatal period, nutrition is of fundamental importance for the proper development of the human organism. A boundless amount of scientific literature in recent years, which is still growing, agrees that breast milk alone is capable not only of satisfying all the nutritional requirements of the newborn and infant in the first months of life but also of being a very important factor in promoting every possible health outcome in the short and long term (American Academy of Pediatrics, 2012).

Given this fundamental and incontrovertible evidence, scientific societies and supra-governmental organisations concerned with public health agree in recommending that infants should be fed only breastmilk for the first six months of life and that breastmilk should remain an integral and fundamental part of their diet until at least two years of age and beyond (American Academy of Pediatrics, 2012; WHO, 2003).

Breast milk, species-specific, has in fact evolved over the millennia hand in hand with mankind, refining nutritional and functional properties that are unattainable for any of its alternatives. Indeed, in its absence, infant formulae, the only other possible alternative, fail to replicate its exact nutritional composition, both in terms of nutrient content and, in any case, in terms of the quality of the nutrients it contains (Grulee et al., 1935). Even more obvious, however, is the impossibility of replicating the functional and bioactive properties of breast milk. And it is precisely the multitude of functional and bioactive compounds in breast milk, which act synergistically with each other in yet unknown pathways, that account for the innumerable benefits of breast milk, evident in both the short and long term.

The benefits of breast milk and breastfeeding

The benefits of breast milk and breastfeeding are being researched by numerous researchers working in various areas of human health. In fact, the literature agrees that to date we have not yet discovered and listed all the benefits of breastfeeding, both nutritionally, functionally and in terms of well-being for the dyad (Dieterich et al., 2013).

What is now well established is that breastfeeding has positive consequences for the health of both mother and baby, both in the short and long term, with the effect depending strictly on both the amount of milk taken in and the duration of breastfeeding, in a clearly dose-dependent manner (Lackey et al., 2021; WHO, 2003).

The benefits to the mother are well known, and their effect increases proportionally with the duration of breastfeeding, but also with the number of children breastfed over a lifetime and thus, with the total number of months of breastfeeding over a woman's lifetime. These

long-term health benefits are mainly in the prevention of certain cancer pathologies such as breast cancer and ovarian cancer but are not limited to this (Stordal, 2022). Prolonged breastfeeding, in fact, also contributes as a protective factor against the development of dysmetabolic diseases such as type 2 diabetes mellitus, but also obesity and high blood pressure (Binns et al., 2016). In the short term, in the first months of breastfeeding, the benefits for the mother mainly concern a lower risk of developing anxiety-depressive syndrome in the post-partum period, precisely because of the response of the oxytocinergic axis that acts both on bonding and on the act of breastfeeding itself (Kendall-Tackett, 2015). Furthermore, exclusive, and prolonged breastfeeding promotes a faster return to pre-pregnancy weight and better metabolic control (Gunderson, 2014). As far as women's health in developing countries is concerned, it is important to remember that prolonged breastfeeding promotes a longer period of amenorrhoea, due to the action of prolactin, which helps to distance pregnancies and thus reduces the risk of premature birth and foetal underdevelopment, while helping mothers to restore the micronutrient reserves necessary for successful pregnancy (Chao, 1987).

The benefits of breastfeeding for the infant can also be divided into short-term and long-term. In the short term, the very nature of breastmilk, especially in the early colostral phase, sees among its pre-eminent functions the protection of the infant against infection, improved gastro-intestinal tolerance to enteral feeding and more effective maturation of the intestine, with reduced intestinal permeability (Binns et al., 2016; K. M. Hurley et al., 2016). Breast milk is also the most important immunomodulatory factor for the newborn, both due to its content of prebiotics, chemokines, and cytokines, and also due to its microbiome and

virome, which in turn modulate and positively influence the development of the neonatal microbiota (Morniroli et al., 2021a). In general, breastfed infants have a lower incidence of infectious diseases and hospitalisation for the first few years of life (Størdal et al., 2017).

The long-term benefits of breastfeeding for the newborn child are now well established, even at later ages up to adulthood (Dieterich et al., 2013). Indeed, with a dose-dependent effect, breast milk is a protective factor against the development of dysmetabolic diseases, obesity, high blood pressure, but also against immune dysregulation diseases such as dermatitis, respiratory allergies, and asthma. Furthermore, again with a dose-dependent effect, mother's milk promotes the best possible development of the child, improving neurodevelopment and IQ at a distance (E. B. Isaacs et al., 2010).

In view of what has just been described and evaluating the beneficial effects that persist into later life and into adulthood, it can be safely stated that breast milk is the first, but also the most important and effective intervention to protect and promote human health.

The complex variability of breast milk

An intrinsic and certainly fascinating feature of breast milk is its variability and ability to modulate in response to both environmental factors and the baby's needs.

Nutritionally, breast milk is able to adapt to the different needs of different categories of infants. In fact, it is now well known that the breast milk of women who have given birth prematurely is richer in protein, compensating, even if only partially, for the increased protein requirements of this population of infants in their first weeks of life (Ballard & Morrow, 2013). Even in full-term infants, however, breast milk changes as the days and

weeks of lactation pass. During the first few days of post-partum, in fact, the mother's milk is called colostrum, a substance produced by the breast from the last weeks of pregnancy, characterised by a content of vitamins, essential fatty acids, and above all, protein that is unequalled in any other phase of lactation (Polidori et al., 2022). Colostrum is in fact very rich in anti-infective substances, which protect the newborn baby from the attack of pathogens in its first days of life. Colostrum contains a large quantity of antibodies, both IgG from the mother's adaptive immunity and, above all, IgA, which, with their cross-reactivity, are able to act against a large quantity of pathogens (Atyeo & Alter, 2021; W. L. Hurley & Theil, 2011). Colostrum is also rich in numerous other functional substances, such as long-chain polyunsaturated fatty acids (LCPUFA) (Bzikowska-Jura et al., 2019), mother's milk oligosaccharides (HMOs), and mucins, which are known for their anti-infective activity (Bode, 2020).

With the initiation of lactogenesis II and the closing of the tigh-junctions at the base of the lactocytes, the colostrum modifies itself by increasing the amount of water, lactose, and more generally, nutrients until, at the end of the first or second week of life, it reaches the final composition of mature milk, which will remain as such, except for some fluctuations in its protein profile, which changes during the course of lactation in step with the development of the infant's gastrointestinal and immune systems (Neville et al., 2001).

The nutritional composition of breast milk is also able to change during the same feeding and throughout the day. In particular, milk produced during the first part of the day contains more lactose and, therefore, for osmotic production reasons, more water, thus having a greater volume. In the afternoon and evening hours, on the other hand, the milk

contains more fat and therefore has a smaller volume per feeding, but of equal caloric value given the higher fat content (Italianer et al., 2020; Khan et al., 2013; Leghi et al., 2021). Even within a single feed, the amount of lactose and fat is not equal. The milk emitted with the first feeding reflex, so-called 'foremilk', is in fact richer in lactose and thus in water, with greater thirst-quenching power. The milk that reaches the baby in the second part of the feeding, known as 'hindmilk', on the other hand, is richer in fat, more satiating, and obviously just as important for a complete and balanced supply of nutrients (Nielsen et al., 2017).

In recent years, an area of interest for researchers has been the ability of milk to change its content throughout the day, not only in terms of nutrients but also in terms of functional substances. This unique characteristic of breast milk, known as 'chrononutrition', is an effective example of how evolution has allowed this food to adapt to the infant's needs (Caba-Flores et al., 2022). In fact, recent studies have shown that in the early hours of the day, in parallel with what happens in the mother's body, breast milk contains a higher amount of cortisol. Towards the middle hours of the day, mother's milk seems to possess higher amounts of immune substances such as antibodies and complement proteins. In the afternoon and evening hours, on the other hand, again in relation to what occurs in the mother, breast milk contains higher amounts of tryptophan first and melatonin later. From this true biological clock of the mother's milk, it is easy to see how breastfeeding is a factor that contributes greatly to the formation of the infant's so-called circadian rhythm and of a sleep-wake rhythm more similar to that of the mother. shared with all other species of diurnal mammals (Hahn-Holbrook et al., 2019).

On the other hand, although breast milk is a variable system that is closely related to what happens in the mother's body, it is equally true that evolution has always prioritised the health and well-being of the offspring. For this reason, even in cases of mild or moderate maternal malnutrition, breast milk retains its nutritional properties and thus remains the best choice for feeding the infant, especially in developing countries and in contexts of resource scarcity (Bravi et al., 2016). Notwithstanding this, studies have shown that the micronutrient content of breastmilk may instead reflect the mother's status and intake of vitamins and minerals, especially zinc and, to a lesser extent, iron (Keikha et al., 2021). Therefore, once again, it is important to emphasise that the health and nutritional status of the mother, as well as in pregnancy, are also important in lactation. Correct planning of the mother's diet, particularly in the case of vegan or particularly restrictive diets, is essential to preserving the adequacy of the mother's milk and thus infant health.

The mother-infant signalling

Communication between a mother and her baby undoubtedly begins in the early stages of gestation. Through the placental circle, the foetus is connected to and dependent on the mother's body for most of its functions and draws oxygen, nutrients, hormones, and substances necessary for its development. This close connection, however, is not limited to the sharing of nutrients but also to a whole series of microorganisms and bioactive compounds, the functions of which are still being studied. For instance, contrary to previous assumptions, we now know that the intrauterine environment is not sterile but has its own specific microbiota that can influence the successful continuation of pregnancy (Moore &

Townsend, 2019). Similarly, oligosaccharides, the prebiotic substances of excellence in breast milk, have also been found in amniotic fluid, testifying to a communication and immunomodulation that takes place well before birth (Wise et al., 2018). In fact, the maternal body, through bioactive compounds, abundance or scarcity of nutrients and hormones, is able to communicate to the developing foetus certain 'information' about the environment that will await it after birth, thus contributing to the development of certain characteristics, both positive and negative, that the foetus will then present once it is born. This epigenetic 'programming' effect continues after birth through the compounds found in the mother's milk, which continue the dyad's communication that began during pregnancy (Hartwig et al., 2017).

On the one hand, this communication undoubtedly has positive implications, as, for example, in the case of foetal stem cells entering the maternal circulation and supporting the maternal organism in protecting itself from tissue damage, ensuring the best possible continuation of the pregnancy itself (Sunami et al., 2010). After birth, the stem cells derived from the maternal mammary gland become part of the cellular compartment of the mother's milk and thus reach the newborn, passing unharmed through the intestinal barrier and entering the neonatal circulation, only to 'die out' after contributing their growth factors to organ development and the repair of any sites of organ damage in the newborn (Hassiotou et al., 2012). This virtuous circle of signalling between mother and child (Figure 2), however, does not always have such positive effects as those just mentioned.

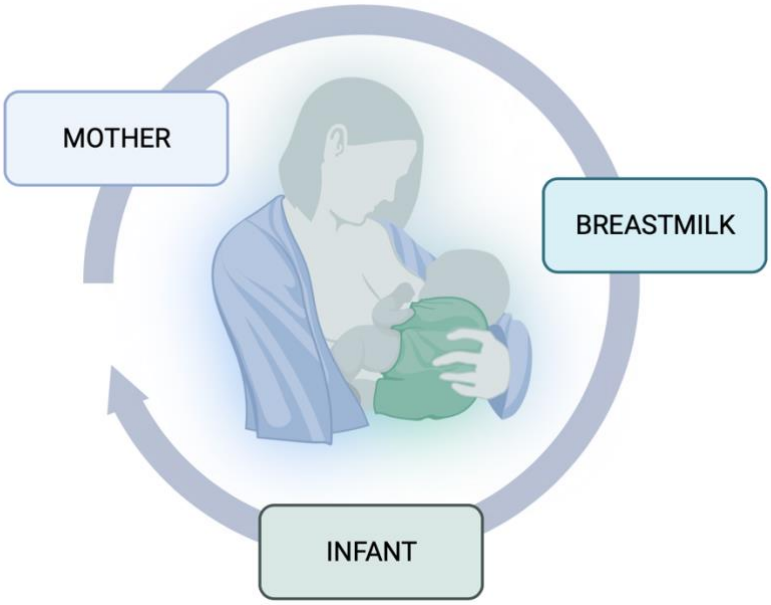

Figure 2. Mother-Infant signalling begins in utero and continues through breastfeeding. Created with Biorender.com

In fact, precisely in the context of preparing the foetus and the newborn for a possible hostile environment, certain environmental situations can activate a stress hormonal response in the mother, which is communicated to the newborn. A study by Grey et al. showed how the presence of increased amounts of cortisol in the mother's milk, derived from a maternal hormonal stress response, can negatively influence infant temperament (Grey et al., 2013). In addition, a review by Di Benedetto et al. analysed the available literature on changes in breast milk in the case of maternal anxious-depressive illness, concluding that changes in the content of certain molecules in breast milk may contribute to the alterations in temperament and cognitive impairment found in children born to anxious and depressed mothers (Di Benedetto et al., 2020).

In conclusion, breast milk can be considered a medium through which the mother's body continues to communicate with the infant after birth, providing information about the

surrounding environment, pathogens in the environment, and contributing to the infant's development in order to make it better prepared for life outside the womb.

1.2 The functional compounds of human milk

As previously described, the importance of breast milk is not limited to its nutritional characteristics, which are perfectly aligned with the needs of the newborn. In fact, much of the benefits of breast milk derive from its very high content of bioactive substances (Ballard & Morrow, 2013) and compounds with functional activity, as shown in Figure 3.

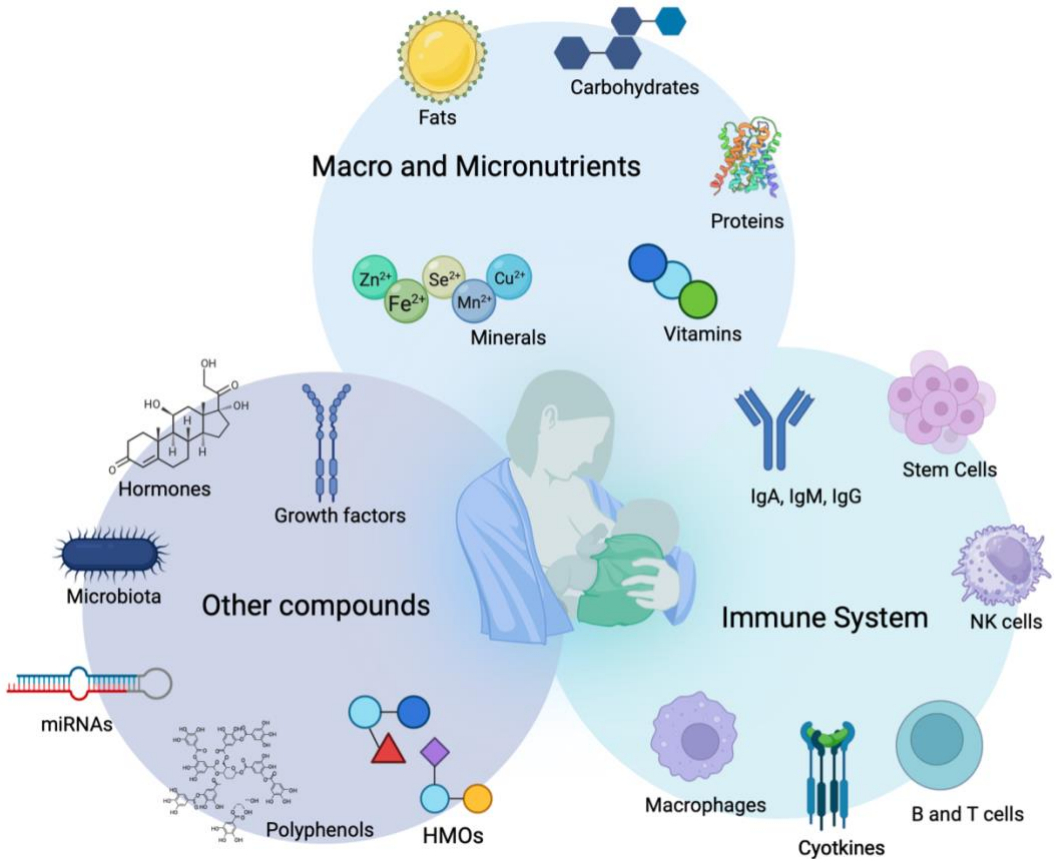

Figure 3. Bioactive compounds of human milk (Modified from Caba-Flores et al., 2022). Created with BioRender.com

An example of this fundamental function of human milk, in addition to its purely nutritional one, is well explored and explained in a paper by Vorbach et al. (Vorbach et al., 2006).

In this paper, the researchers focused on trying to understand what the ontogenic evolution of the mammary gland might have been, concluding that it probably evolved from a tegumental mucous gland. In fact, the mammary gland would have retained its characteristics in terms of secretion of anti-infectious and protective substances, to which other substances with a more attractive nutritional function would only have been added later. Although this is speculation at present, albeit likely, the work of Vorbach et al. gives a good idea of how breast milk, in its composition of bioactive substances, expresses its very important role in protecting the newborn and promoting its health.

The dual role of nutrients in breast milk

Analysing the nutritional components of breastmilk, such as protein, alpha-lactalbumin is the most important serum protein in breastmilk. In fact, alpha-lactalbumin has been reported to exhibit antimicrobial properties against bacteria such as Streptococcus pneumoniae, Haemophilus influenzae, and enteropathogenic strains (Hakansson et al., 2000; Layman et al., 2018; Lobb et al., 2023). In particular, certain peptides derived from its enzymatic digestion, pentapeptides linked by a disulfide bridge, appear to be responsible for its antimicrobial activity against pathogens.

As for lipids in breast milk, recent studies show their antimicrobial activity.

Lipids in human milk have been demonstrated to exhibit both antiviral and antibacterial activity. For this reason, they have been studied as additional components in infant formulas

in order to exploit not only their nutritional but also their anti-infective and functional properties (C. E. Isaacs et al., 1990). As explained in a review by Isaacs, the antimicrobial activity of breast milk lipids appears to be exerted by the medium-chain fatty acids and long-chain polyunsaturated fatty acids, as well as their respective monoglycerides, released during the enzymatic digestion process in the gastrointestinal tract of the newborn (C. E. Isaacs, 2001).

The Milk Fat Globule Membrane (MFGM) is a vital component of human milk that encapsulates fat globules. It plays several essential roles in infant nutrition and development.

This complex structure presents a three-layer membrane surrounding a triglyceride-rich core. It is a crucial element in the nutritional composition of breast milk, providing infants with a well-balanced and easily digestible source of nutrients and bioactive compounds essential for their growth and development. From a nutritional point of view, MFGM promote the emulsification of fats, making them more easily digested and absorbed by the infant's developing digestive system (Chai et al., 2022). However, MFGM's role is not limited to providing lipids; in fact, considering the variety of membrane proteins present in the lipid layers, they act as carriers of various bioactive compounds, including lipids and glycoproteins such as lactoferrin, ensuring their efficient delivery to the infant.

From an immunological viewpoint, MFGM contain immunoglobulins, glycoproteins, and phospholipids that contribute to the immune properties of breast milk, strengthening the baby's immune system (Lee et al., 2018; Manoni et al., 2020).

Recent studies suggest that certain components of MFGM, such as sphingolipids and gangliosides, may promote cognitive development in infants and have anti-inflammatory properties (Lee et al., 2018; Pan et al., 2023). This may be particularly helpful in reducing inflammation and promoting gut health in children. Research indicates that MFGM components may influence the composition of the gut microbiota, contributing to a healthy gut environment in infants (Zhao et al., 2022). Finally, MFGM play a role in metabolic programming, influencing metabolic pathways in infants, with long-term effects on health (Ye et al., 2021).

There is also a proven functional capacity with regard to the sugars in human milk, which constitute its major component. Indeed, breastmilk oligosaccharides are a key component of breastmilk sugars, and their very important role in the health of the newborn will be discussed in the later chapters of this dissertation. Additionally, lactose itself, a major component of breast milk, has been studied for its functional role. Lactose, the most represented component of breast milk, has been investigated for its influence on neonatal immunity. Studies suggest that lactose plays a role in augmenting the immune defense of infants, contributing to their innate immune system. It is considered not only as a nutritional source but also as a factor that enhances the immune response in neonates (Cederlund et al., 2013).

Leucocytes, cytokines and immunomodulating compounds

The infant's immune system at birth is known to be immature. The neo-natal immune response is in fact based mainly on innate immunity, whereas adaptive immunity is

ineffective, with an imbalance towards the Th2 response, which is antibody-oriented and is typical of allergic diseases, to the detriment of the Th1 response and the differentiation of B-type lymphocytes (Adkins et al., 2004).

This, together with the relative immaturity of the infant's organs and apparatuses and its naivety towards pathogens, account for the immaturity and inadequacy of the neonatal immune system. In this context, mother's milk becomes a source of substances that can positively stimulate and modulate the newborn immune system, fostering its maturation.

A fundamental element of the immunising properties of breast milk is precisely its immune cell content, shown in Figure 4 (Witkowska-Zimny & Kaminska-El-Hassan, 2017).

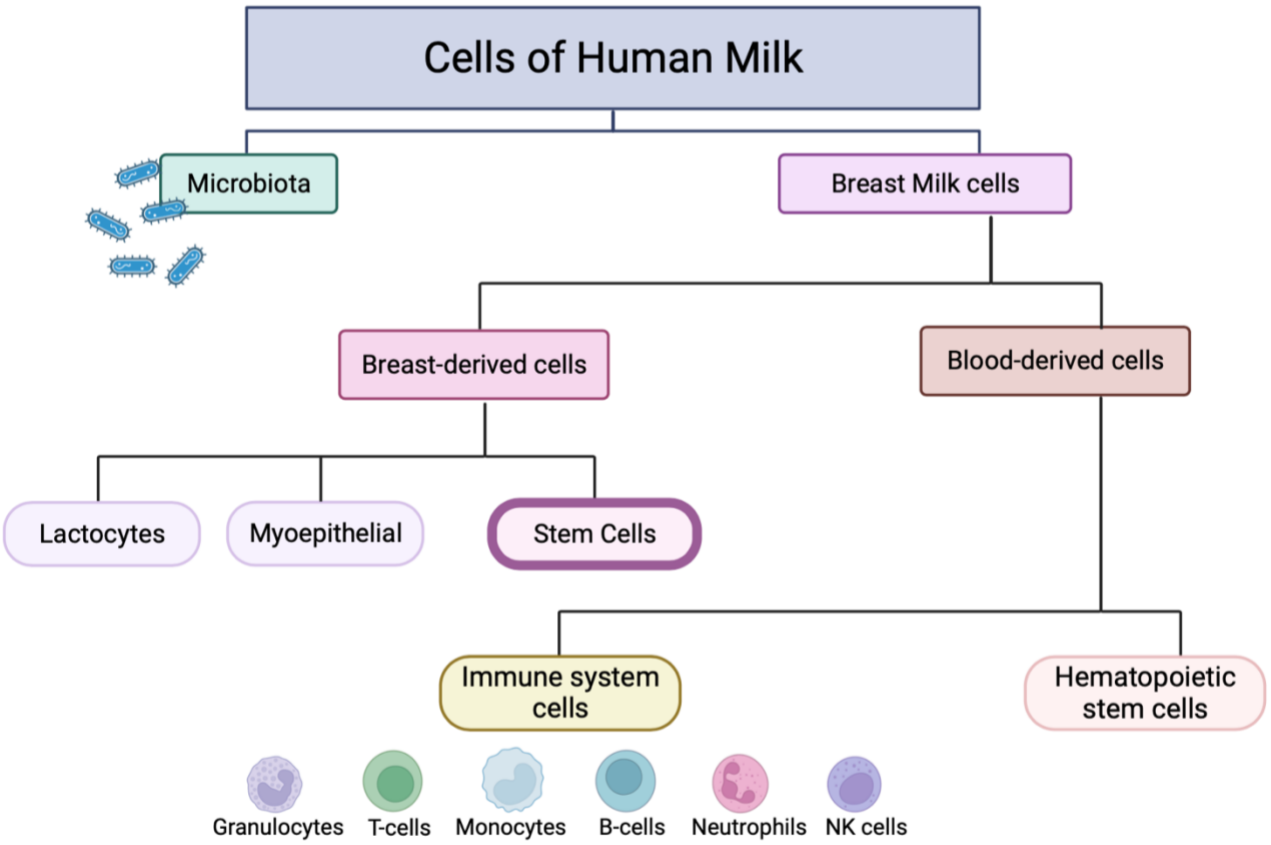

Figure 4. Cells of human milk. Created with Biorender.com

Breast milk leucocytes are more abundant in colostrum and then decrease in the first few days of post-partum. They consist, for the most part, of about 80 percent of macrophages. As an illustration of the dyad communication that takes place through breast milk, it is worth mentioning how an entero-mammary transport circle of leucocytes, originating in the mother's intestinal mucosa-associated immune system (MALT), has been assumed to be in response to stimuli and pathogens she encounters (Laouar, 2020). Through this entero-mammary communication, leukocytes are then transferred into the mother's milk and later into the infant's intestine. Once they cross the neonatal intestinal barrier, the leukocytes of breast milk localise in the Peyer's plaques along the neonatal intestine, regulating its development and supporting its function. In addition, breast milk leucocytes have also been identified in the newborn's lymph nodes, spleen, and liver (Cabinian et al., 2016). Once inside the infant's body, breast leucocytes have the task of modulating and regulating the T-lymphocyte response, probably by enhancing CD8+ T-lymphocytes in dealing with intracellular pathogens, suppressing immature cytotoxic T-lymphocytes, and at the same time reducing the T-helper response to good bacteria in the developing microbiota (Lokossou et al., 2022).

Cytokines are also present in varying amounts in breast milk, both pro-inflammatory and anti-inflammatory cytokines. The pro-inflammatory cytokines in breast milk, in particular TNFα, IL-6, IL-8, and IFNγ, are responsible for stimulating neonatal immunity and defending against pathogens. TGF-β, also present in breast milk, is responsible for reducing inflammation, stimulating tissue repair, and hindering the development of allergies (Kiełbasa et al., 2021).

Interleukins are also well represented in breast milk, if IL-8 promotes chemotaxis of neutrophils, IL-1 also present in milk attenuates IL-8 activation and suppresses pro-inflammatory responses of nuclear factor kappa beta (NF-kB) signalling (Nolan et al., 2019). A cytokine highly represented in breast milk is transforming growth factor beta 2 (TGF-β2). Its abundant presence in breast milk promotes intestinal maturation, production of immunoglobulins by the neonatal immune system and T-lymphocyte suppression. Its presence has also been associated with the development of a more favourable gut microbiota. Given its functions, its content in breast milk appears to be a protective factor against the development of necrotising enterocolitis (NEC) (Rautava et al., 2011).

A functional protein in breast milk, lactoferrin, has also gained importance in recent years for its immune role. In particular, researchers have focused on its antiviral properties, discussed later in this dissertation; however, its most prominent action is antibacterial. In fact, the lactoferrin of mother's milk, once digested in the stomach, is transformed into lactoferricin, a compound capable of neutralising the endotoxins of possible intestinal pathogens, but also capable of modulating the intestinal inflammatory response, limiting the action of pro-inflammatory cytokines and, more generally, reducing intestinal inflammation, as shown in studies that have tested its supplementation in inflammatory bowel diseases of various kinds (Albenzio et al., 2016; Campione et al., 2020; Chen et al., 2017).

Considering the compounds in breast milk with an immunomodulatory function, great importance in terms of both quantity and importance should be given to immunogobulins. In particular, in addition to G-class and M-class immunoglobulins that have a passive

transmission function of immunity, the immunomodulating part is played by class A immunoglobulins (IgA), and in particular by secretory IgA (W. L. Hurley & Theil, 2011). Secretory immunoglobulin A is produced once IgA, produced by mammary cells, is cleaved into IgAs and then transported into the milk compartment.

The total amount of sIgA, understandably, is especially high in colostrum and in the first days of lactation, then decreases in mature milk but still remains one of the most important functional protein components.

The function of secretory IgA is crucial in protecting the infant from respiratory and intestinal infections but is not limited to this. Indeed, in addition to hindering bacterial adhesion to the intestinal wall, this form of immunoglobulin promotes the development of a favourable microbiota and promotes gut-associated lymphoid tissue (GALT) (Donaldson et al., 2018; Dunne-Castagna et al., 2020). The IgAs content of breast milk is influenced by maternal lifestyle habits, with exposure to smoking and alcohol reducing the amount. Moreover, the levels of IgA have been observed to be diminished in the mature milk of mothers who undergo gestational diabetes, postpartum stress, anxiety, and depression (Schneider et al., 2017). Conversely, mothers with atopic or allergic symptoms or frequent infections have a higher sIgA content (Mulyani et al., 2023). Furthermore, the content and type of IgA in breast milk appears to be influenced in turn by the maternal gut microbiota. There is a strong correlation between the presence of elevated levels of IgA, cytokines, oligosaccharides, and various other immune factors in human milk and a decreased likelihood of developing food allergies during early childhood (Van De Perre, P, 2003).

Human milk and the microbiota

Breast milk is certainly the most important factor promoting the development of a favourable microbiota in the newborn. Given its value in promoting the proliferation of 'beneficial' gut bacteria, it has also been regarded as the infant's first true prebiotic food.

The development of the gut microbiota probably already occurs in utero (Moore & Townsend, 2019). In fact, the latest scientific evidence shows that at the placental level and in the amniotic fluid, the uterine environment is not sterile but already contains at least a portion of bacteria probably derived from the maternal intestinal microbiota. Other studies show that while it is still unclear whether the infant's microbiota develops already in the foetal period based on the amniotic microbiota, there is also evidence that by-products of the bacterial presence reach the foetal circulation (Gil et al., 2020).

In particular, recent work by Wise et al. has shown that breastmilk oligosaccharides, compounds with strong immunomodulatory and prebiotic activity, have also been found not only in the maternal circulation but also in the amniotic fluid, although their role and value in this compartment have yet to be studied and demonstrated (Wise et al., 2018).

The maternal microbiota during pregnancy is influenced by several factors, such as lifestyle, diet, genetics, geographic location, health status, and most importantly, antibiotic use (Cheng et al., 2022; Sinha et al., 2023). Once again, providing correct information to pregnant women proves to be an effective strategy for positively modulating both maternal and newborn health outcomes.

During birth, the use of antibiotic drugs and the mode of delivery influence the newborn microbiota. It is now well known that delivery by caesarean section hinders the creation of

a positive microbiota, as the mother's vaginal and intestinal microbiota cannot colonise the infant as it does not pass through the birth canal (Hoang et al., 2021). To overcome this discrepancy, there have been numerous studies proposing various methods of 'administering' a positive microbiota to the newborn, but no method to date has proved to have proven and, above all, lasting efficacy (Wong et al., 2022). While it has now been demonstrated that the microbiota of Caesarean section babies tends to conform to that of vaginal birth babies during the first year of life, it is also true that the type of feeding greatly influences changes in the microbiota, both positively and negatively (Coscia et al., 2021). In this sense, formula feeding, particularly if it is exclusive, compromises the development of a desirable microbiota, regardless of the type of delivery. In contrast, breastfeeding can promote a favourable microbiome even in infants born by caesarean section or who have undergone early antibiotic therapy (Guo et al., 2020).

This positive effect of breast milk on the microbiota depends on several factors, first and foremost the microbiota of breast milk itself. Breast milk, in fact, contains bacteria at a concentration of about 1,000 colony-forming units per millilitre (CFU/mL), the ranks of which have been identified in recent studies and which are likely to be derived from the mother's gut via that entero-mammary circulation that has already been discussed above (Cabrera-Rubio et al., 2016). The main identified strains are Bifidobacterium, Lactobacillus, Enterococcus, and Staphylococcus, which are also found in the first faeces of breastfed infants. Some studies have suggested that commensal bacteria present on the mother's skin surface also enter the infant's oral cavity and then, via a retrograde flow of milk, have the opportunity to spread and replicate in the maternal alveoli (Biagi et al., 2018). However, this

explanation does not account for the diversity and plurality of strains present in breast milk, including more than just commensal strains of the epidermis. The role of the microbiota of breast milk is not only that of 'first colonisation' of the neonatal intestine, but rather, some Lactobacillus strains isolated in breast milk have a true and proper competitive action and inhibition of the adhesion of pathogens to the intestinal surface of the infant, probably by stimulating the production of mucins by the intestinal cells, thus having a protective action against the development of diseases (Jara et al., 2011).

The milk microbiota, however, is not only composed of bacteria. In fact, the virome of breast milk has also gained great interest in research in recent years. This population of'resident' viruses found in breast milk does not include pathogenic viruses, which are instead found in milk in cases of maternal infection and illness. The viroma of breast milk consists mainly of phages, which, upon reaching the infant's gut, feed on the mycobacteria, contributing to the control of the bacterial population and positively regulating the infant's microbiota (Mohandas & Pannaraj, 2020).

The positive effect of breast milk on the gut microbiota is not limited to the direct presence of microorganisms that can regulate and colonise it (Lim et al., 2015).

In fact, as mentioned above, breast milk can be defined as a true prebiotic in view of the large quantity of substances present that have precisely this function. Lactose, the main sugar in breast milk, serves not only as an energy feeder for the infant but also for the microbiota. In fact, strains of bifidobacteria and lactobacilli, which are good for human health, feed on this sugar, and its abundance encourages their proliferation. Certain nucleotides contained in breast milk, molecules from which DNA and RNA with multiple

other functions are synthesised, have also been studied as being able to positively influence the microbiota (Qu et al., 2023). A study by Singhal et al. showed that their supplementation is able to increase the proportion of bifidobacteria in the newborn' intestine (Singhal et al., 2008). Their role as prebiotics, however, remains a matter of debate (Mha & Shamir, 2000). Lactoferrin, a protein with the multiple properties mentioned above, has also been shown in some studies to have the ability to influence the microbiota. While it promotes bacterial antagonism and prevents the overgrowth of pathogenic bacteria, it also appears to have a true prebiotic effect on certain strains of Bifidobacteria and Lactobacilli, as demonstrated in a study by Chen et al. (Chen et al., 2017)

Extracellular vesicles (EVs) are small membrane-bound structures released by cells into their environment. They play a crucial role in intercellular communication and are involved in various biological processes. The main type of EVs in human milk are exosomes (Galley & Besner, 2020). Extracellular vesicles (EVs) play a significant role in the formation of the infant microbiota. Research suggests that EVs in breast milk are able to transport genetic material and microRNAs in the gut and influence the resident microbial population (Galley & Besner, 2020; Turunen et al., 2023).

Indeed, they may facilitate the transfer of important biological molecules and genetic material, influencing the constitution of the early gut microbiota.

In summary, extracellular vesicles are emerging as key players in the creation and development of the infant's gut microbiota, potentially influencing the early colonisation of beneficial bacteria, and also contributing to the overall health and well-being of the child (Kim & Yi, 2020a).

Last but certainly not least, an important prebiotic in breast milk is breast milk oligosaccharides (HMOs). These compounds, which have a relatively simple molecular structure and great variability, are a fundamental constituent of breast milk. In fact, they are present in a concentration that varies from 5 to 15 g/dL, much more than the proteins themselves in breast milk. This large quantity is certainly an indication of their importance in terms of promoting infant health (Bode, 2020).

In fact, HMOs have been the subject of much research and a large body of scientific literature in recent years. Among their many functions, some of which are still unclarified and some of which will be discussed in the next chapters of this dissertation, their role as prebiotics certainly stands out.

In fact, HMOs serve precisely as 'food' for gut bacteria, promoting their replication and the production of short-chain fatty acids (SCFAs), which in turn are beneficial for the immune regulation of the gut. On the other hand, they can also act as decoys for pathogenic bacteria, preventing their cell adhesion and thus the development of disease, and instead stimulating intestinal cell proliferation and improving the epithelial barrier (Walsh et al., 2020).

Growth factors, enzymes and nucleotides

Human breast milk is rich in various growth factors that play a crucial role in a child's development. These bioactive compounds promote the growth and development of various tissues and organs in the newborn (Galante et al., 2020).

Epidermal growth factor (EGF) is essential for the growth and maturation of the gastrointestinal tract in infants. It contributes to the development of the intestinal mucosa,

promoting nutrient absorption and protection from pathogens. Heparin-binding epidermal growth factor (HB-EGF) has also been found in breast milk. Its role is to promote tissue regeneration and general gut repair. Recent studies have shown that its presence in breast milk, like that of EGF, is a protective factor against the development of NEC (Oguchi et al., 1997; Patki et al., 2012). Another key factor in the diversity of breast milk is lactadherin. This glycoprotein is present in the lipid bilayer of fat globule membranes and has the fundamental task of promoting phagocytosis of apoptotic cells, helping to maintain intestinal homeostasis and the proper barrier function of the intestinal mucosa. It also increases the effectiveness of the tight junctions at the base of intestinal cells, helping to reduce intestinal permeability. Finally, it plays an anti-inflammatory role, reducing the effect of pro-inflammatory cytokines and other mediators of inflammation. It has therefore also been identified as a protective factor against NEC (Newburg et al., 1998; Zhou et al., 2010).

IGF-1, or insulin-like growth factor-1, is critical for general growth, including skeletal growth, tissue repair, and the development of the central nervous system of the newborn. It therefore promotes the development of various organs and tissues in the child, but its action is comparable to that of a hormone, which will be discussed later (Serrao et al., 2016). Nerve growth factor (NGF) is important for the development and maintenance of the nervous system and promotes the development, proliferation, and survival of nerve cells (Kim & Yi, 2020).

Platelet-derived growth factor (PDGF) is involved in various cellular processes, including growth, division, and angiogenesis (the formation of new blood vessels). It contributes to

tissue repair and development and is also implicated in protective factors in NEC and other inflammatory bowel diseases (Oguchi et al., 1997).

Transforming growth factor beta, or TGF-β, plays a crucial role in immune regulation and tissue repair. It helps maintain a balanced immune response and promotes tissue healing (Oguchi et al., 1997). The role of TGF-β2 in modulating the infant's immune system has been discussed above.

The most important enzyme in breast milk is certainly lysozyme. Its effect is mainly antimicrobial; it has a strong bactericidal action and, in combination with lactoferrin, has the ability to bind bacterial lipopolysaccharide (LPS), preventing pathogens from attaching to the intestinal mucosa. Its function in controlling intestinal infectious diseases is also enhanced by the fact that the Paneth's cells themselves, intercalated in the lining of the intestinal mucosa, produce this enzyme to protect the mucosa (Chantry et al., 2011).

Apart from lysozyme, the enzymes in human milk are manifold and have mainly a function in promoting digestion. In fact, in breast milk, we can find amylase, lipase, glycogenase, protease, and nucleotidase, as well as numerous other enzymes capable of degrading a wide variety of compounds, such as nucleic acids, mucins, and others (Khaldi et al., 2014). An interesting enzyme in breast milk is catalase, which has the task of degrading hydrogen peroxide and thus has an important antioxidant function, as will be discussed later.

Nucleotides are crucial intracellular compounds that play various roles in biological processes. Their role in modulating the infant's microbiome has been previously discussed. It is not entirely clear whether the nucleotides found in human milk result from the degradation of nucleic acids or if they are actively secreted in response to specific stimuli.

Research indicates that, expressed as nucleotide equivalents, they are present in human milk in different forms, including nucleic acid, nucleotides, and nucleosides in the context of human milk, serve a specific function (Hodgkinson et al., 2022).

These nucleotides include adenosine monophosphate (AMP), guanosine monophosphate (GMP), cytidine monophosphate (CMP), and uridine monophosphate (UMP), among others and their concentrations vary within a range of 0% to 7.38% of the nonprotein nitrogen fraction of breast milk

Human milk's nucleotides have been suggested to have a potential role in sleep induction and sleep homeostasis, aiding in the regulation of sleep patterns in infants (Sánchez et al., 2009).

Human milk as antioxidant

The antioxidant properties of human milk are of utmost importance in protecting infants from numerous diseases. Indeed, reactive oxygen species (ROS) have a significant impact on cell signalling processes, yet their presence and especially their excess, given their powerful oxidising potential, can cause cell damage.

Breast milk contains a large number of antioxidants, the full list of which is certainly not yet known. Antioxidants play a key role in protecting infants from oxidative damage and can be classified as exogenous or endogenous (Aceti et al., 2018). Exogenous antioxidants are those compounds that are obtained from the diet, the content of which in breast milk is therefore also influenced by the maternal diet. These include polyphenols, carotenoids, and vitamins.

Polyphenols, as well as other phenolic compounds, are a group of naturally occurring antioxidants found in plants. They have been identified in human milk, and their content is obviously influenced by the amount taken in with the mother's milk.

Vitamins A, C and E are well-known antioxidants and are present in human milk. They help protect cells from damage caused by free radicals and contribute to the infant's immune system development (Lorenzetti et al., 2021).

Carotenoids are natural pigments found in plants and some microorganisms. The most known are beta-carotene, lutein, and zeaxanthin, well represented in human milk and contribute to its antioxidant capacity. They are important for visual development and immune function in infants (Lorenzetti et al., 2021).

In contrast, endogenous antioxidants in breast milk include specific molecules, such as certain enzymes such as catalases and superoxide dismutase. Their task is to react with certain chemical compounds and molecules, cancelling out their oxidative potency. (Silvestre et al., 2008).

Glutathione is also an important antioxidant in breast milk and is produced by the body itself. Glutathione is a tripeptide, i.e., it consists of three amino acids: glutamic acid, cysteine, and glycine. Its composition gives it the ability to oxidise or reduce itself, acting as an alternative target for oxidising substances and circulating free radicals, thus protecting cells from oxidative damage (Xavier et al., 2011).

Melatonin, the hormone that promotes sleep and circadian rhythm development, has also been studied for its antioxidant power, acting as a scavenger, and promoting the expression

of certain antioxidant enzymes such as catalase and glutathione peroxidase (Reiter et al., 1999)

Overall, Breast-fed infants demonstrate a significant reduction in oxidative stress, as evidenced by the presence of fewer biomarkers of oxidative damage than formula-fed infants (Silvestre et al., 2008).

Hormones in human milk

In recent years, the hormone content of breast milk has attracted great interest. Researchers have wondered whether the presence of certain digestive hormones or regulators of appetite and metabolism underlies the different feeding behaviour of the breastfed infant compared to the formula-fed infant. In fact, the breastfed infant has greater satiety, generally consuming less milk for the same number of calories. Furthermore, numerous studies in the literature have shown that the breastfed infant has a higher lean mass intake and a lower percentage of fat mass than the formula-fed infant. In an attempt to explain this phenomenon, researchers have also focused on the hormones in breast milk. In fact, human milk contains some hormones derived from the maternal blood cycle. While the role of some of these hormones, such as cortisol and melatonin, has been well established, as explained above, and is comparable to the effect they have on adults, for other hormones, literary data are still strongly conflicting (Mazzocchi et al., 2019).

Leptin, ghrelin, insulin growth factor 1 (IGF-1), adiponectin, and insulin are among the key factors that significantly influence growth, body composition, and the long-term risk of obesity in adulthood (Uysal et al., 2002). Leptin is involved in regulating appetite and

energy expenditure. Ghrelin is also a hunger hormone that stimulates appetite. It helps regulate feeding behaviour in infants and may influence their growth patterns. However, research results on the correlation between ghrelin levels in breast milk and the rate of weight gain in infants have been diverse and conflicting. IGF-1 plays a crucial role in regulating cell proliferation and preventing apoptosis (Kon et al., 2014). It has been observed that infants with significant weight gain consume breast milk with elevated levels of IGF-1; however, these findings are not consistent across studies (Kon et al., 2014; Mazzocchi et al., 2019). To date, the role of IGF-1 in weight gain and the apposition of fat and lean mass in the newborn remains debated.

Breast milk also contains other hormones, including adiponectin, known to be an anti-inflammatory hormone and to increase insulin sensitivity. However, human milk also contains resistin, a hormone that has the opposite function of increasing insulin resistance. Its role in the health and growth of the infant is still unknown (Suwaydi et al., 2021).

Finally, breast milk also contains a quantity of insulin. This hormone, as is already known, is degraded at the gastrointestinal level, and therefore its presence in milk does not seem to influence the glucose homeostasis of the newborn. However, some experimental studies have suggested that insulin taken orally may have a trophic effect on the intestinal wall (Shehadeh et al., 2006). To date, as with other glucose metabolism hormones present in breast milk, the role of insulin in breast milk is unknown. Research continues to try to answer these questions and fully understand what role these hormones may play in the development of metabolism and body composition in newborns.

Stem cells, exosomes and miRNAs

One of the most fascinating discoveries concerning the potential of the human milk is certainly the presence of stem cells. Stem cells from breast milk are still being studied, but it is now clear that they are pluripotent cells. In numerous studies, these cells express markers such as nestin, cytokeratin, OCT 4, SOX 2, NANOG, SSEA 4, and TRA, indicating their potential to differentiate into various cell lineages (Mane et al., 2022).

On the role and final fate of breast milk cells, the work of Foteini Hassiotou was of fundamental importance (Hassiotou et al., 2013). Her research, first in experimental models and then in vivo, showed that the stem cells, once they have passed unharmed through the infant's gastric digestion, locate themselves in the intestine of the newborn and then cross it and enter the blood stream (Twigger et al., 2013). In the past, some studies had suggested that these pluripotent cells then go on to localise in target organs and apparatuses, differentiate into the various cell lines, and proliferate with the tissue. If this hypothesis had been confirmed, it would have been interesting to consider breastfed infants as carriers of true microchimerism, as they contain cell lines from a different genetic lineage, the maternal one (Molès et al., 2018).

More recent studies have instead suggested that the fate of breast milk stem cells is to contribute to tissue repair and regeneration, potentially aiding in the healing process, but not through their proliferation. Indeed, it has been assumed that once they reach the target organ, these cells go on to exert a mainly paracrine function, meaning they can secrete factors that influence neighbouring cells. This secretion of bioactive molecules, rather than

their proliferation, plays a role in tissue repair and modulating local cellular environments (Ninkina et al., 2019).

It's important to note that while the presence of stem cells in breast milk is an exciting area of research, further studies are needed to fully understand their precise mechanisms and long-term effects on neonatal health. However, it is also important to note that, once again, breast milk is a living food whose therapeutic potential is clearly superior to any other food. Moreover, recent studies have shown that breast milk cells, once isolated, have the ability to be reprogrammed 'in vitro' to differentiate into other tissues (Mane et al., 2022). While research in this area is still ongoing, the discovery of stem cells in breast milk holds promise for potential therapeutic applications in the future. They could potentially be used in regenerative medicine and tissue engineering.

Exosomes are extracellular vesicles secreted by various cells and composed mainly of a lipid bilayer. Through the transfer of exosomes from one cell to another, not only genetic material (RNAm, DNA, and miRNA) but also proteins and lipids necessary for the cell to survive and perform its functions are transferred (Admyre et al., 2007).

Generally, stem cells have the task of acting to regenerate the organ in which they are located. As already described, stem cells from breast milk enter the infant's circulation and reach the target organs. Human milk stem cells regenerate the target organ tissue by releasing exosomes that will promote cell replication of healthy adult cells, which in turn will have specific receptors for exosomes (Kersin & Özek, 2021).

Independent of stem cells, human milk transfers substantial amounts of microRNAs that are responsible for numerous functions through exosomes.

The roles of exosomes in human milk are diverse and vital to infant development. These tiny vesicles play a significant role in intercellular communication and the transportation of bioactive molecules. Exosomes in human milk contain a rich cargo of genetic material, including microRNAs, mRNAs, and proteins, which have been shown to exert various biological activities (Feng et al., 2021).

Research suggests that exosomes may influence immune responses and modulate inflammation in infants. They carry immune modulatory features, potentially contributing to the development and regulation of the infant's immune system (Admyre et al., 2007). Moreover, studies indicate that these exosomes may have myocardial reparative functions, which could play a crucial role in cardiovascular health. Furthermore, exosomes in human milk might impact the infant's digestive system. They are thought to participate in gut maturation and may have implications for the establishment of a healthy gut microbiome. Exosomes may also support neurodevelopment, as they contain bioactive molecules that could potentially influence brain development and function (Admyre et al., 2007; Feng et al., 2021).

MicroRNAs are a group of small, non-coding RNAs with a single-stranded RNAs that have been identified in many organisms They consist of 18–22 nucleotides. Their fundamental role is to negatively regulate gene expression at the post-transcriptional level. Indeed, miRNAs act by recognising specific mRNA targets to determine their degradation or repression of gene translation (Alsaweed et al., 2015).

However, miRNAs are not only found inside cells but can also be transported outside them and are found in numerous biological fluids, where they can be used as extracellular diagnostic markers for the diagnosis and treatment of certain diseases (Condrat et al., 2020). Circulating miRNAs in biological fluids, such as those found in breast milk, represent a new form of intercellular communication through the transfer of genetic information from a sending cell to a receiving cell. It is now known that the miRNAs present in milk are not found in their free form but are transported by extracellular vesicles, including the exosomes just described.

An extensive review by Ahlberg et al. identified in the literature the targets of the 10 most found miRNA types in breast milk. From this review, it appears that the main systems and apparatuses of miRNAs in breast milk are the immune system, with regulatory functions, and the gastrointestinal system, for the induction of food tolerance and the prevention of allergies (Ahlberg et al., 2023).

Furthermore, miRNAs in human milk may exert epigenetic effects. They have the potential to influence specific gene expression patterns throughout an individual's life, impacting long-term health outcomes. This epigenetic regulation through miRNAs could have far-reaching implications for the infant's development, not only in terms of immune function but also in other physiological processes (Chuang & Jones, 2007).

The study of miRNAs in breast milk is still ongoing, and certainly in the coming years research will yield interesting new insights into their functions and their therapeutic potential in disease.

1.3 The potential of human milk as an antiviral agent

It is now well known that breast milk can protect infants from viral infections. Indeed, its ability to protect infants from gastro-intestinal viral infections makes its prolonged use recommended both in developing countries and in the event of disasters and natural disasters (WHO, 2003).

But not only that, in a position statement on breastfeeding by the American Academy of Paediatrics (AAP), it is pointed out that numerous studies in the literature show that breast milk is able to prevent viral infections in infants (American Academy of Pediatrics, 2012).

A study on the epidemiology of breastfeeding published in Lancet, by Victora et al. on behalf of the Lancet Breastfeeding Series Group, confirmed these evidences (Table 1).

Outcome	**Mode of breastfeeding**	**OR (95% IC)**
Mortality secondary to infectious disease in the first six months	Exclusive vs predominant	OR: 0.68 (0.52; 0.88)
	Exclusive vs partial	OR: 0.35 (0.20; 0.61)
	Exclusive vs none	OR: 0.07 (0.03; 0.16)
Mortality secondary to infectious disease in the first 6-23 months	Any vs none	OR: 0.48 (0.38; 0.60)
Infectious diseases		
Diarrhoea age < 5 years	More versus less Breastfeeding (e.g. exclusive versus non-exclusive; predominant versus partial; partial versus none; any BF versus no BF, etc.)	RR: 0.69 (0.58;0.82)
Diarrhoea age < 6 months		RR: 0.37 (0.27; 0.50)
Diarrhoea age 6 months-5 years		RR: 0.46 (0.28; 0.78)
Hospitalisation for diarrhoea age < 5 years		RR: 0.28 (0.16;0.50)
Lower respiratory tract infections < 2 years	More versus less Breastfeeding	RR: 0.68 (0.60; 0.77)

Hospitalisation for lower respiratory tract infections < 2 years	(e.g. exclusive versus non-exclusive; predominant versus partial; partial versus none; any BF versus no BF, etc.)	RR: 0.43 (0.33; 0.55)
Otitis media < 2 years		OR : 0.67 (0.62, 0.72

Table 1. Benefits of breastfeeding on paediatric infectious disease. OR: odds ratio; RR: risk ratio. Modified from Victora CG et al. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. Lancet. 2016 Jan 30;387(10017):475-90 (Victora et al., 2016).

Over the years, the antiviral components of breastmilk have attracted increasing interest, and certainly, ongoing research in this regard has led to numerous discoveries, some of which are still being studied.

Thanks to the numerous data points in the literature, several components of breast milk are now known to exert antiviral activity. If we want to classify these substances, they can be listed in three large groups: cells of the immune compartment, substances belonging to the adaptive immune response, and finally, substances belonging to innate immunity (Morniroli et al., 2021a).

Regarding the immune system cells present in breast milk, as described above, these are mainly characterised by macrophages, followed by neutrophils, and lastly by lymphocytes. To a lesser extent, but still present in breast milk, we also find natural killer (NK) cells and mast cells. The contribution of the immune cell compartment in the defence of the body against viruses is certainly important and reflects the activities and functions that these cells habitually have in the body(Witkowska-Zimny & Kaminska-El-Hassan, 2017).

The adaptive immune response of breast milk is mainly composed of antibodies. In breast milk, the main antibodies responsible for antiviral activity are IgA, IgG, and IgM (Rio-Aige et al., 2021).

IgA is the most abundant immunoglobulin in human milk, playing a crucial role in providing passive immunity to infants, especially on the mucosal surfaces of the respiratory and gastrointestinal tracts, helping to protect against infections (W. L. Hurley & Theil, 2011). As discussed above, it is mainly secretory IgA that makes up most of the immunoglobulins in milk.

Interestingly, although IgA is produced by B lymphocytes, or plasma cells, in response to a specific pathogen, some studies have shown an ability to cross-react with viruses appearing in the same family or sharing similar receptors or surface structures. This characteristic makes them very important in protecting the newborn against viral infections. In fact, by settling on the gastrointestinal and respiratory mucous membranes, they are able to protect against certain viruses but also against related viruses.

Although found in lower concentrations compared to IgA, IgG is another significant immunoglobulin present in human milk. It provides systemic immunity and enhances the overall immune protection of the infant. IgM is less abundant than IgA and IgG but is also present in human milk. It contributes to the immune defence of the infant against various pathogens. The IgG and IgM found in breast milk derive from the maternal bloodstream and are therefore a direct expression of the pathogens that the nursing mother has encountered in the past or is currently fighting (Rio-Aige et al., 2021). This seems crucial if we consider that, given the proximity of the dyad, it is difficult to think of a pathogen reaching only the infant and not the mother. Although the newborn has a still immature immune system, the more competent mother's body mounts the immune response to the pathogen that the dyad has encountered and rapidly initiates the transfer of antibodies via

milk, thus providing a true passive immunisation for the newborn, which is crucial in reducing and positively modifying the infant's disease course (Rio-Aige et al., 2021).

This antibody transmission between mother and newborn child is the basis of the numerous campaigns that have been carried out worldwide to encourage nursing mothers to carry out the necessary vaccinations, especially during breastfeeding. Indeed, apart from a few exceptions concerning certain attenuated vaccines, such as the BCG vaccine against tuberculosis, yellow fever, and Japanese encephalitis, all other vaccines are not only possible during pregnancy but are even strongly recommended if the mother discovers that she is not protected against one or more vaccine-preventable diseases (Brady et al., 2018).

A clear example of this phenomenon was what happened during the SARS-CoV-2 pandemic. Once an adequate and effective vaccine against this virus had been created and produced on a large scale, lactating mothers were also invited to receive the planned vaccine doses in order to protect themselves and their newborn child against this new virus.

Numerous studies carried out during the vaccination campaign showed that the milk of breastfeeding mothers who had received the SARS-Cov-2 vaccine contained varying proportions of IgA and IgG antibodies directed against the virus. These antibodies were also detected in the mother's milk for up to six weeks after the vaccination had been carried out, thus guaranteeing the newborn important and, above all, long-lasting protection through passive immunisation (Bianchi et al., 2022). Interesting work by Bode et al. showed how the response to vaccination by the nursing mothers, although it occurred in all subjects, was characterised by IgA and IgG directed against various portions and subunits of the virus, with some mothers also showing cross-reacting antibodies to other human coronaviruses

and others not showing this characteristic (Bode et al., 2023). Future studies are certainly needed to understand the reasons behind this important difference in the type of antibodies produced by mothers and found in breast milk.

The third large group of substances in breast milk with antiviral properties are the compounds of innate immunity. This is undoubtedly the largest but also the most heterogeneous group, including substances typically characteristic of the immune response, such as cytokines and complement proteins, but also a multitude of substances not directly involved in the immune system. The latter include large molecules that hinder viral adhesion to cells, such as mucins and glycosaminoglycans, but also HMOs, nucleotides, lactadherin, lactoferrin and other functional proteins in breast milk, and the lipid fraction with LCPUFAs, MCTs and mono- and diglycerides (Morniroli et al., 2021b).

This list is certainly not exhaustive, as the study of the antiviral components of breast milk leads to frequent and exciting discoveries of new substances with interesting functions. And it is also interesting to mention that even regarding already well-known antiviral compounds, their action is still not fully known but only assumed. Many researchers agree that these numerous substances probably act synergistically, making it even more difficult to understand their mechanism of action and their exploitation as compounds to be supplemented for human health.

In conclusion, the study of functional components of human milk is a fascinating and expanding field of research. Future studies using modern metabolomics, mass spectrometry, lipidomics, and proteomics techniques are therefore needed to answer the

many questions about the nature of these molecules and especially their mechanism of action. A greater understanding of these aspects would lead not only to a broadening of knowledge about breast milk but also to the possibility of isolating these compounds and exploiting them for prevention and the promotion of human health at all stages of life.

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Chapter 2

Human milk as an innate antiviral agent against SARS-CoV-2

Part of the research findings described in this chapter have been previously published in: **Morniroli D**, Signorini L, Dolci M, Vizzari G, Ronchi A, Pietrasanta C, Pugni L, Mosca F, Delbue S, Gianni ML. Breastmilk from COVID-19 negative lactating mothers shows neutralizing activity against SARS-COV-2. Sci Rep. 2023 Sep 19;13(1):15521.

2.1. Research background and aim of the study

On March 11, 2020, the WHO declared pandemic status for SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2), or COVID-19 disease (the Coronavirus Disease of 2019) (Cucinotta & Vanelli, 2020).

The exact source of the virus is still under scrutiny, but it's thought to have zoonotic origins, meaning it likely originated in animals before spilling over to humans. The initial cases were linked to a seafood market in Wuhan, China, in late 2019, where live wild animals were also sold (Pagani et al., 2023).

Scientific research has indicated that the virus is genetically related to coronaviruses found in bats, which suggests that bats could be a natural reservoir for similar viruses. However, the exact intermediate species or route through which the virus was transmitted to humans is still an area of active investigation.

Italy was one of the first countries affected, and certainly northern Italy was one of the hardest-hit regions. In late February 2020, the first known local transmission of COVID-19 occurred in Lombardy, Northern Italy, with cases reported in towns such as Codogno and Castiglione d'Adda. In March 2020, the virus rapidly spread across Italy, leading to a sharp

increase in cases. The outbreak was particularly severe in the Lombardy, Emilia-Romagna, and Veneto regions (Sebastiani et al., 2020).

On March 9, 2020, the Italian government implemented a nationwide lockdown to curb the spread of the virus. The coverage of an effective vaccine against this virus has certainly changed the course of pandemics, saving millions of lives worldwide. Italy initiated a widespread vaccination campaign starting in late December 2020. The vaccination rollout aimed to protect the population and achieve herd immunity.

SARS-CoV-2 is an RNA virus belonging to the Coronavirus family that is already known to cause illness in humans, ranging from the common cold to more severe respiratory diseases such as MERS (Middle East respiratory syndrome) and SARS (Severe Acute Respiratory Syndrome)(Wang et al., 2020).

Coronaviruses are so named because of their appearance under electron microscopy, where they display an envelope dotted with numerous spikes, the S-glycoprotein or 'Spike' protein, which gives them a crown-like appearance (Figure 5) (Wang et al., 2020).

Three S-glycoproteins joined together make up a trimer; the trimers of this protein form the structures that, as a whole, resemble a crown surrounding the virion. The main differences between this new coronavirus and the SARS virus seem to lie in this spike protein. The S-glycoprotein is the one that determines the virus' specificity for the epithelial cells of the respiratory tract; the 3D model in fact suggests that SARS-CoV-2 is able to bind the ACE2 (angiotensin converting enzyme 2) receptor, which is expressed by the capillary cells of the respiratory system (Wang et al., 2020).

SARS-CoV-2 has undergone various mutations. These mutations involve changes in its genetic code, which then affect certain membrane protein modifications. Notable mutations have been observed near the S1/S2 cleavage site and in non-spike regions (Abbasian et al., 2023). Mutations are common in RNA viruses like SARS-CoV-2, but not all mutations significantly alter the virus's characteristics.

Research continues to delve into the genetic variations of SARS-CoV-2, providing valuable information for regional surveillance and global health efforts. These studies aim to unravel the virus's evolution and its potential implications for public health.

The entry of SARS-CoV-2 into host cells is a critical step in the infection process. This virus relies mainly on the interaction between its spike protein (S) and the receptor of the angiotensin-converting enzyme 2 (ACE2) present on the surface of human cells. This interaction initiates membrane fusion, allowing the virus to enter the host cell. The spike protein undergoes proteolytic cleavage, facilitating the fusion of viral and cell membranes (Jackson et al., 2022).

Enveloped viruses face several obstacles in their attempt to enter host cells, including physical barriers, the host's immune response, and the intricate cellular processes they must pass through. These mechanisms collectively serve as the host organism's defence mechanism against viral infection.

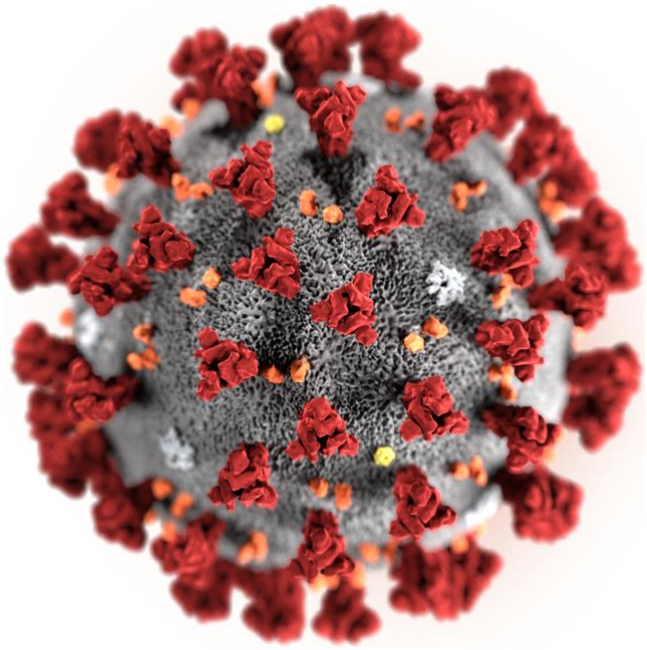

Figure 5. Created by the US Centers for Disease Control and Prevention (CDC), the image reveals the ultrastructural morphology of SARS-CoV-2. The S-glycoproteins (in red) are visible on the surface, giving it the appearance of a crown. Credits to: https://phil.cdc.gov/Details.aspx?pid=23312

Since the beginning of the pandemic, researchers around the world have joined efforts in an attempt to develop a vaccine as quickly as possible, but also to find molecules or compounds that could reduce the chance of falling ill or mitigate the course of the disease.

Among the many possibilities explored, certain compounds contained in breast milk, such as oligosaccharides (HMOs) and lactoferrin, have been extensively studied.

In particular, great attention has been paid to lactoferrin. This breastmilk protein, with promising antimicrobial capabilities that have been discussed previously, has been studied for its superficial ability to hinder virus entry into cells by multiple mechanisms. Lactoferrin

has also been the subject of numerous clinical trials that have attempted to explore its role in both preventing infection by SARS-CoV-2 and modulating the disease course of already infected patients (Bolat et al., 2022).

Oligosaccharides in breast milk have also been studied has a potential as a barrier to viral entry. Breast milk oligosaccharides, particularly the two subtypes most studied for their antiviral role, are molecules containing sialic acid residues. Human milk sialylated oligosaccharides (HMOs) have demonstrated antiviral properties and may play a protective role against viral infections, including those caused by respiratory viruses (Chutipongtanate et al., 2022).

Sialylated compounds such as HMOs and certain types of glycoproteins have been studied in the literature for their role in inhibiting viral entry into cells. Indeed, viral recognition of sialic acid has been recognised as a pathogenicity factor for several infections. However, sialoma may also play a role in protecting against viral infections. As a host defence mechanism, O-linked sialylated glycans covering mucins on the mucosal cell surface form a thick layer of sialylated residues that act as a barrier, preventing pathogens from entering the cell by providing an alternative binding site (Morniroli et al., 2020).

An interesting study by Varki et al. hypothesised, considering the type of sialylated residues typical of human beings, that the importance of sialic acid in viral infections underlies this difference (Varki, 2009). In particular, Varki postulated that a viral pandemic, caused by a pathogen with a pronounced tropism for N-glycolylneuraminic acid (NeuGc) sialylated residues, eliminated the human population with that particular residue, instead causing individuals that synthesised exclusively N-Acetyl-Neuraminic Acid (NeuAc) to survive,

thus leading to this substantial difference between the sialylated residues found on the cells of human beings and those that line the cell surface of species phylogenetically closer to humans.

Long-chain polyunsaturated fatty acids (LCPUFAs) have also been described as having a certain protective capacity against respiratory infections, albeit by an as yet unknown mechanism (De Cosmi et al., 2022). In addition, omega-3 supplementation has been investigated as a possible prevention for the development of COVID-19 disease, whereas omega-3 deficiency in the body has been linked to a more severe disease course in several studies (Mazidimoradi et al., 2022).

However, research on these substances and their role in the prevention of viral diseases, including COVID-19, is still ongoing. Although the results of some studies appear promising, the clear role of these substances, both when naturally present in the body and as external supplementation, has yet to be defined.

Considering these assumptions and knowing that the presence of non-specific antiviral substances in breast milk is well known, a study was conducted to assess the antiviral capacity of breast milk against SARS-Cov-2 in seronegative women who lacked breastmilk antibodies against the virus.

2.2 Study population and methods

From November 2020 to May 2022, we screened SARS-CoV-2 positive pregnant women admitted to the maternity ward of a third-level care hospital and referral centre. All women were checked for SARS-CoV-2 presence in a nasopharyngeal swab before to hospital admission for birth and then directed to the well-baby delivery room and nursery or the designated COVID-19 maternity unit. Following enrollment, women were split into two groups according on their infectious status: COVID-19 positive moms and negative and unvaccinated mothers. Group B moms said that they had never had a prior positive swab and had not been vaccinated, either by choice or because they were recruited in the trial during the early months (final trimester of 2020 and early 2021), when immunisation was not accessible to the general public. Exclusion criteria for group B moms included a prior positive SARS-CoV-2 swab or COVID-19 infection at any point in their clinical history, as well as having received even a single dose of SARS-CoV-2 vaccination. However, it should be noted that the moms in Group B may have previously had an asymptomatic illness that was unknown to them and hence not reported. During the mother's hospital stay (up to three days postpartum for negative moms), enrollment took place in the well-baby nursery and the COVID maternity ward. The average age of the moms who participated was 33.6 5. Milk samples were taken at random from women in both groups at different breastfeeding stages: 2 days (T0 - colostrum), 7 days (T1 - transition milk), and 20 days (T2 - mature milk) postpartum (Figure 6). Milk samples were collected in a sterile test container by hand expression at the conclusion of a single nursing or breast pumping session. Prior to the collection of milk samples, all recruited mothers provided written informed permission. The

Ethics Committee "Milano 2" of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico authorised the research, which was carried out in compliance with the Declaration of Helsinki and hospital and municipal applicable government norms and laws. After collection, milk samples were maintained at -80°C before being moved to the Translational Research laboratory, which is fully equipped with a biosafety-level-3 (BSL3) facility.

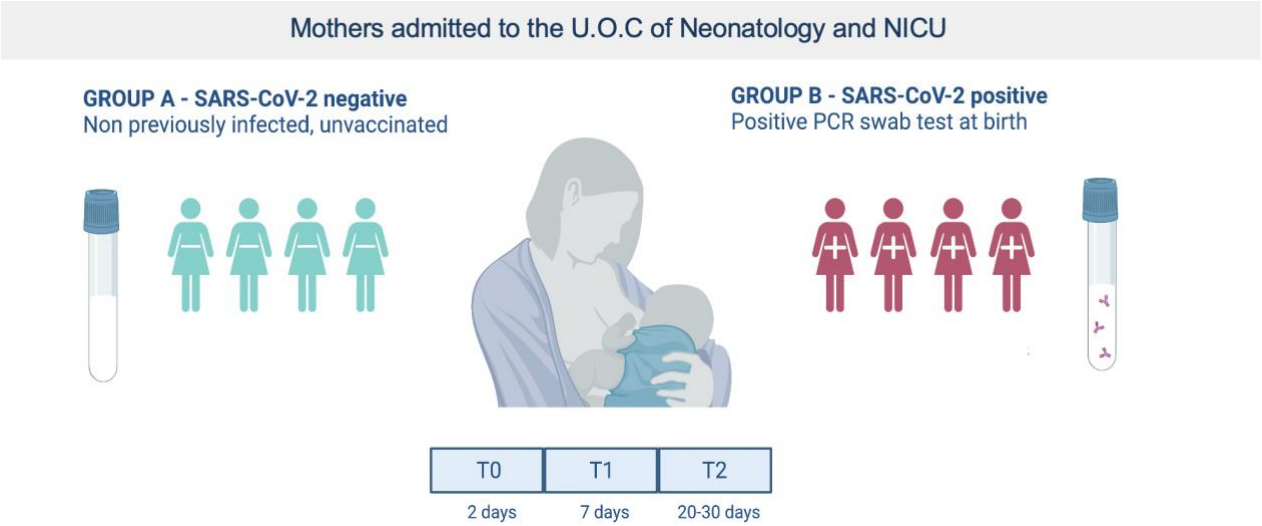

Figure 6. Population enrolled in the study and time-points (Created with BioRender.com)

Assay evaluating microneutralization

In 96-well culture microplates, 15 000 VERO E6 cells (CRL-1586, American Type Culture Collection (ATCC) USA) were seeded and allowed to adhere overnight in complete medium at 37°C in a humidified atmosphere containing 5% CO2 until 80% confluence was reached. Milk samples were heat inactivated for 40 minutes at 56°C before being serially diluted two times in complete medium from 1:2 to 1:1024 in quintuplicate. Following dilution, 200

TCID50 (50% Tissue Culture Infectious Dose) SARS-CoV-2 virions (B.1) (SARS-CoV-2/human/ITA/Milan-UNIMI-1/2020, GenBank MT748758.1) were combined with breast milk dilutions and incubated for 1 hour at 37°C in a humidified environment containing 5% CO2. Milk preparations were seeded on VERO E6 cell monolayers grown overnight on 96-well culture microplates and incubated for two hours at 37°C in a humidified environment containing 5% CO2 after incubation. After that, each well received complete medium and was cultured for 5 days at 37°C in a humidified environment containing 5% CO2. Each plate was examined for cytopathic effects (CPE) using an inverted optical microscope on day 5 after infection. The Reed and Muench method was used to calculate the 50% neutralising endpoint titer (NT50)(Reed & Muench, 1938). The experiment was carried out at a biosafety level 3 (BSL3) facility (Figure 7).

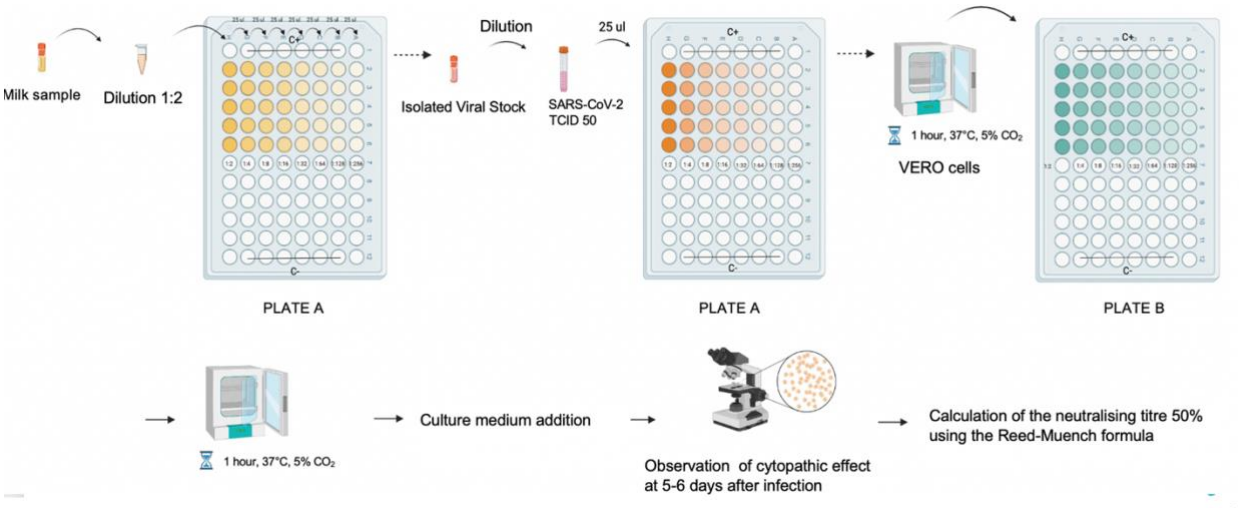

Figure 7. Neutralizing assay determining the cytopathic effect caused by viral replication in VERO cell population placed in culture, incubated and infected with SARS-CoV-2 (Created with BioRender.com)

The SARS CoV-2 virus Assay with NeutraLISA

Following the manufacturer's instructions, the presence and quantity of SARS-CoV-2 specific neutralising antibodies in breast milk samples were assessed using the commercial kit SARS-CoV-2 ELISA test (SARS CoV-2 NeutraLISA, Euroimmun, Italy) (Figure 8). Milk samples were centrifuged for 15 minutes at 1500 rpm. To eliminate or diminish the inhibitory effects of fat on future studies, the creamy layer of fat globules was removed. The milk whey supernatant was then centrifuged at 12,000 rpm for 10 minutes to remove creamy residues before being diluted 1:5 for the ELISA test. In brief, 100 uL of diluted samples were added to the microplate and incubated for one hour at 37°C. After washing each well three times with the kit's washing buffer, 100 uL of enzyme conjugate was added to each well and incubated for 30 minutes at room temperature. Following three washes, 100 uL of chromogen/substrate solution was applied to each well and incubated at room temperature for 15 minutes, protected from light. After stopping the reaction with 100 uL/well of stop solution, the plate was read at 450 nm. Each plate had a blank, positive, and negative control, and each sample was examined in triplicate. The findings were semi-quantitatively reported as a percentage of inhibition (%IH) and analysed as follows: %IH 20: negative, %IH 20 and 35: borderline, and %IH 35: positive. The %IH was computed as follows: %IH = (Sample absorbance x 100%)/(Blanck absorbance):

$$100\% = \frac{Sample\ absorbance\ x\ 100\%}{\text{Blanck absorbance}} = \%\text{IH}.$$

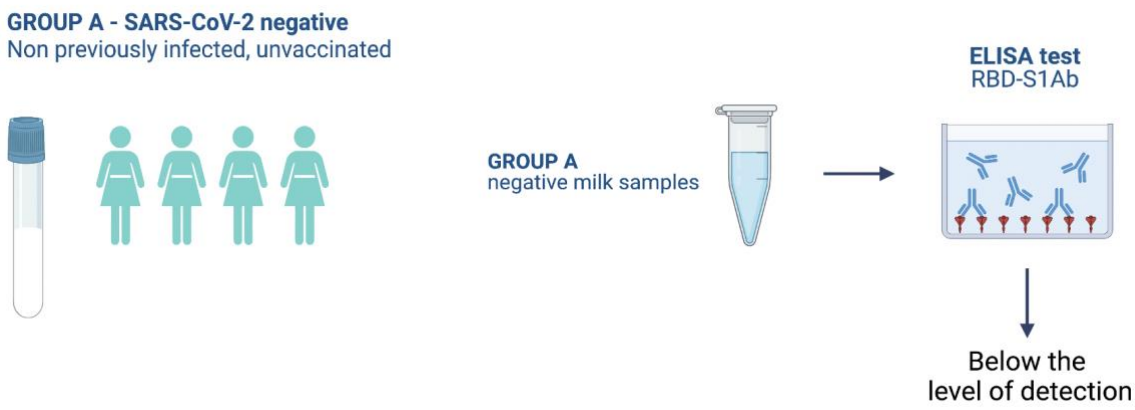

Figure 8. Antibodies essay with SARS-CoV-2 ELISA test SARS CoV-2 NeutraLISA, Euroimmun, Italy (Created with BioRender.com)

Statistical analysis

Statistics were analysed using the SPSS version 21 statistical software programme (SPSS Inc., Chicago, IL, USA), and graphs were created using GraphPad Prism 9 for Windows (GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com). Medians and interquartile ranges (IQR) were used to define categorical and numerical variables. The Mann-Whitney U test and the Kruskal-Wallis nonparametric test were used to compare median NT50 values. Pearson's correlation analysis was utilised to examine the relationship between the %IH and the associated NT50 values.

2.3 Results

Breastmilk NT_{50} titers

We gathered 60 breastmilk samples from the 49 mothers who participated. Group A consisted of fifteen SARS-CoV-2 positive moms and 34 SARS-CoV-2 negative mothers. In groups A and B, 19 and 41 milk samples were taken, respectively. The number of breastmilk samples collected in group A at each time point was as follows: T0: 5 samples, T1: 7 samples, T2: 7 samples (table 2).

	Group A (n=19)			**Group B (n=41)**		
	T0 (n=5)	T1 (n=7)	T2 (n=7)	T0 (n=24)	T1 (n=10)	T2 (n=7)
Median	1:32.0	1:22.8	1:3.2	1:11.3	1:4.0	1:2.8
Min	1:16.0	1:4.0	1:2.3	1:2.8	1:2.3	1:2.3
Max	1:512.0	1:256.0	1:20.7	1:81.6	1:6.3	1:6.3

n = number of milk samples

Table 2. Breastmilk samples NT_{50} values (SARS-CoV-2 B.1 lineage)

Below is the distribution of the number of breastmilk samples taken from each time point in group B: 24 samples were taken at time T0, 10 were taken at time T1, and 7 were taken at time T2 (table 2). The details of the median NT50 levels for each group at each timepoint may be seen in table 2, as well as figures 9 and 10.

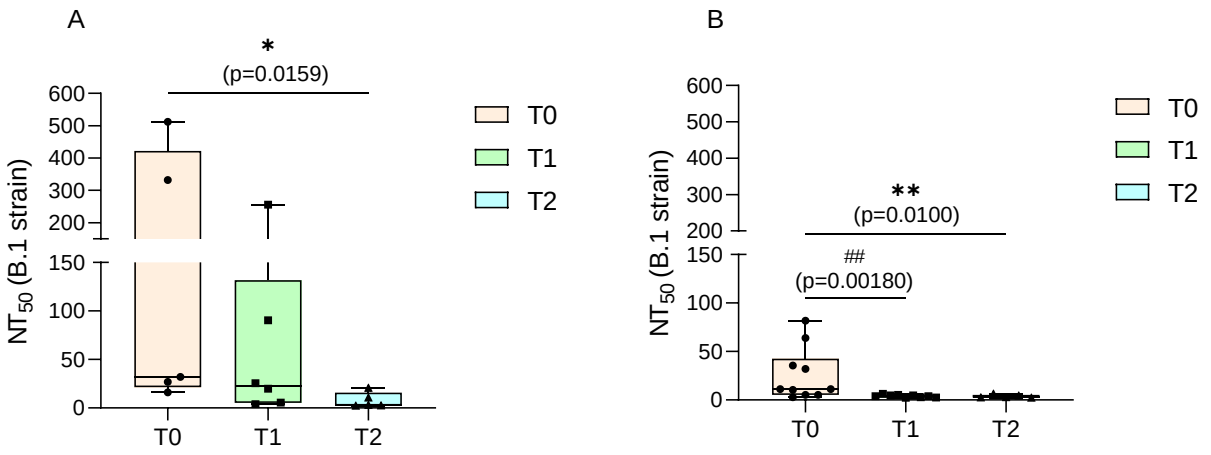

Figure 9. NT_{50} values against SARS-CoV-2 B.1 lineage in group A (panel A) and group B (panel B) in breastmilk samples at each timepoint.

The boxes extend from the 25th to 75th percentiles, plots whiskers down to the minimum and up to the maximum value, each individual value is a point superimposed on the graph and the middle lines of each box shows medians. *p = 0.0159 (panel A), ##p=0.00180 **p=0.0100 (panel B) (statistical test: Mann-Whitney U test).

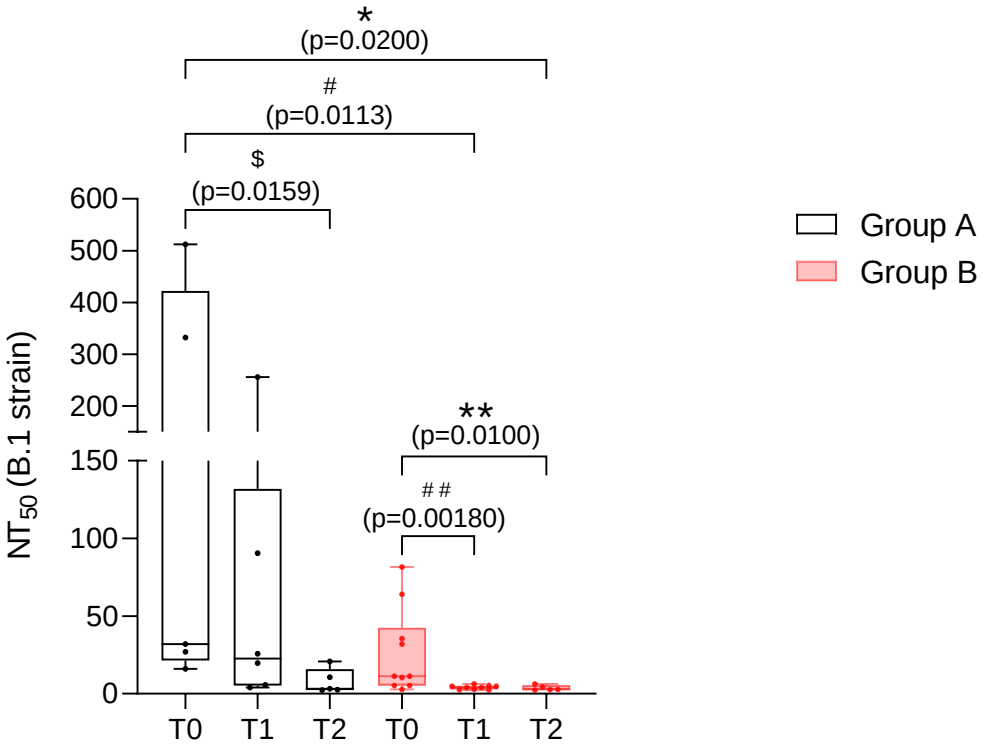

Figure 10. NT_{50} values against SARS-CoV-2 B.1 lineage in group A (white boxes) and group B (red boxes) in breastmilk samples at each timepoint.

The boxes extend from the 25th to 75th percentiles, plots whiskers down to the minimum and up to the maximum value, each individual value is a point superimposed on the graph and the middle lines of each box shows medians. $^{\$}p = 0.0159$, $^{\#\#}p = 0.00180$, $^{**}p=0.0100$(statistical test: Mann-Whitney U test), $^{*}p = 0.0200$, $^{\#}p = 0.0113$ (statistical test: Kruskal-Wallis nonparametric test).

Breastmilk samples from group A exhibited neutralising action against SARS-CoV-2 B.1 lineage in a total of 16 out of 23 (84.21%) samples, whereas breastmilk samples from group B showed activity in 24 out of 41 (58.54%) samples. The time point analysis revealed that in group A, there was neutralising activity in a total of 5/5 samples (100.00%) at T0, 6/7 samples (85.71%) at T1, and 5/7 samples (71.43%) at T2. In group B, there was neutralising activity in 10/24 (41.67%) at T0, 9/10 (90.00%) at T1, and 5/7 (71.43%) at T2. In Group A, the values for

the median NT50 at T0, T1, and T2 were 1:32.00, 1:22.76, and 1:3.21, respectively, whereas in Group B, the values were 1:11.31 at T0, 1:4.00 at T1, and 1:2.83 at T2 (table 1). In group A (figure 1, panel A), significant differences were discovered in the median NT50 values between T0 and T2 ($p = 0.0159$); in group B (figure 1, panel B), significant differences were discovered between T0 and T1 ($p = 0.0018$); and between T0 and T2 ($p = 0.0100$). The comparison of the median NT50 values between group A and group B revealed a statistically significant difference between T0 (group A) and T1 (group B) ($p = 0.0113$) and T0 (group A) and T2 (group B) ($p = 0.0200$) (figure 2).

SARSCoV-2 NeutraLISA ASSAY

In order to determine whether or not milk samples contained neutralising antibodies specific to SARS-CoV-2, a semiquantitative ELISA test designed specifically for SARS-CoV-2 was carried out. It was determined how much breastmilk each of the enrolled women produced throughout each of the several time periods. Due to the limited quantity of breastmilk that was collected, the analysis could not be performed on 2 samples taken at T1 from group A, 7 samples taken at T0, 6 samples taken at T1 and 4 samples taken at T2 from group B. The number of breastmilk samples that were analysed at each time point was as follows for group A: 5 samples were analysed at T0, 5 samples were analysed at T1, and 7 samples were analysed at T2 (table 3).

	Group A (n=17)			**Group B (n=24)**		
	T0 (n=5)	T1 (n=5)	T2 (n=7)	T0 (n=17)	T1 (n=4)	T2 (n=3)
Median	43.4	38.8	35.3	31.2	40.2	23.1
Min	29.1	26.8	21.6	1.8	28.3	13.8
Max	85.7	96.1	44.1	68.1	61.8	49.2

Interpretation of the semiquantitative result: %IH ≤ 20: negative, %IH ≥ 20 and < 35: borderline, %IH ≥ 35: positive. n = number of milk samples.

Table 3. Breastmilk %IH values

Regarding group B, the following is a description of the number of breastmilk samples that were analysed at each time point: 17 samples taken at T0, 4 samples taken at T1, and 3 samples taken at T2 are included in table 2. Breastmilk samples from women in group A demonstrated the presence of SARS-CoV-2 specific neutralising antibodies in a total of 13 of 17 (76.5%), and 10 of 24 (41.7%), respectively, out of the available 24. The time point analysis revealed that in group A, there were a total of 4/5 samples (80.0%) at T0, 3/5 samples (60.0%) at T1, and 4/7 samples (57.1%) at T2 with %IH 35 (figure 3), while in group B, there were a total of 6/17 samples (35.3%) at T0, 3/4 (75.0%) at T1, and 1/3 (33.3%) at T2 with %IH 35 (Figure 11).

The results of Pearson's correlation analysis demonstrated a substantial positive association between %IH and paired NT50 for group A (R2 = 0.7, p 0.0001), whereas there was no correlation for group B (R2 = 0.01951, p = ns) (Figure 12).

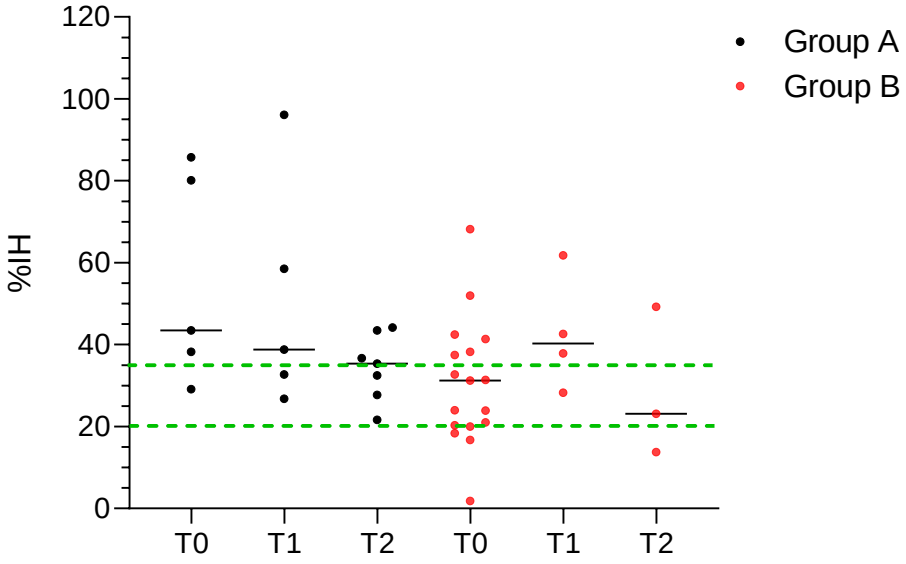

Figure 11. %IH values against SARS-CoV-2 in group A (dark dots) and B (red dots) in breastmilk samples at each timepoint.

The black lines represent the median %IH values. The green dotted lines represent the 20%IH and 35%IH. Interpretation of the semiquantitative result: %IH ≤ 20: negative, %IH ≥ 20 and < 35: borderline, %IH ≥ 35: positive.

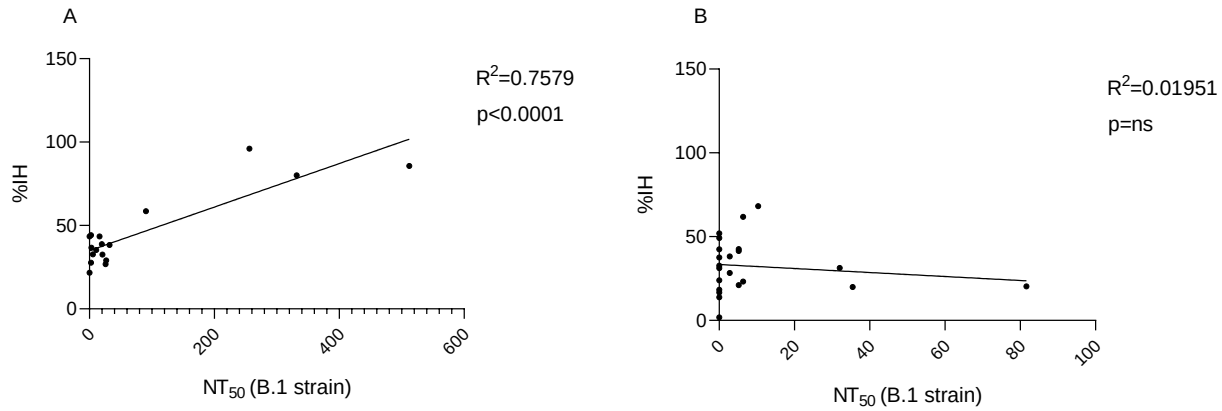

Figure 12. Pearson's correlation between breastmilk %IH and NT_{50} titres. Pearson's correlation analysis showed significant positive correlation in group A (R^2 = 0.7579, p < 0,0001) (panel A) and no correlation in group B (R^2 = 0.01951, p = ns) (panel B).

In order to get a better understanding of what could be the underlying cause of this innate neutralising activity that is expressed by breastmilk, we are going to analyse any differences that exist between the negative samples in terms of the concentration of different fatty acids, particularly LCPUFA, considering the known antiviral activity that these acids already possess against enveloped viruses.

These early observations, which have not yet been published, indicate that the neutralising ability seems to be directly tied to the presence of eicosatrienoic acid (Fugure 13), which is also known as Mead acid. Eicosatrienoic acid is well recognised for its osteoblastic, antitumoral, and anti-inflammatory action.

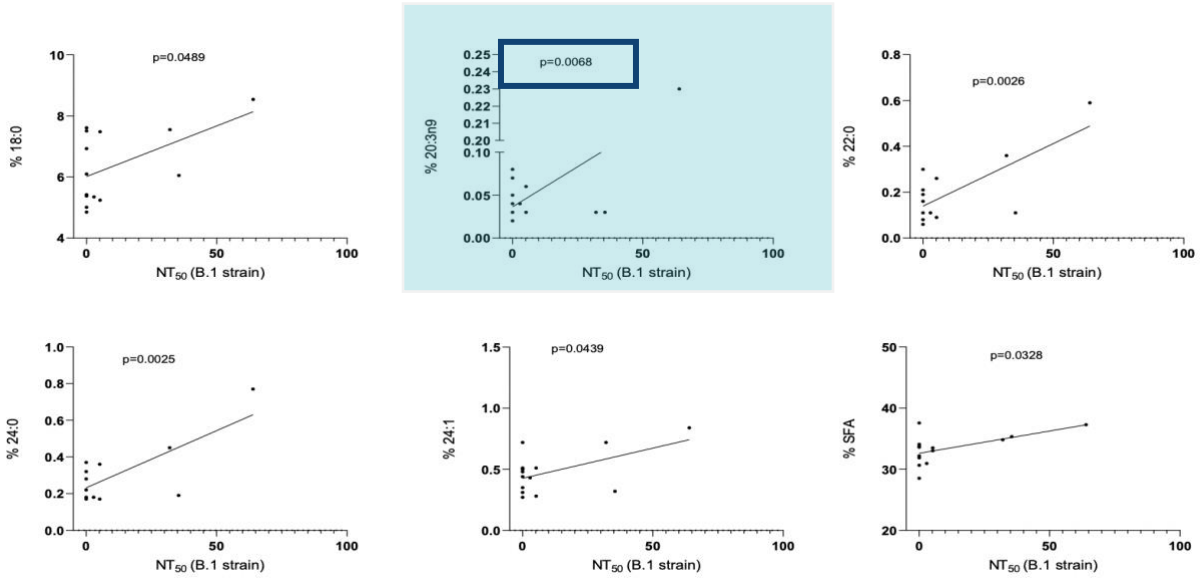

Figure 13. Pearson's correlation analysis between the neutralizing activity (NT_{50}) of breastmilk on the B.1 strain of SARS-CoV-2 and the percentage of different fatty acids in breastmilk. The eicosatrienoic acid (20:3n9) shows a significant correlation (p= 0.0068).

In order to further explore the possible reasons underlying this innate antiviral aptitude, further laboratory tests were conducted on HMOs. In order to verify which concentration of HMOs corresponded to the levels of inhibition shown by breast milk, the amounts of viral particles produced by infected cells treated with different concentrations of oligosaccharide were titrated (Figure 14). In particular, a search for the SARS-CoV-2 genome (copies/mL) was conducted in the supernatants of infected cells treated with different concentrations of a mixture of synthetic oligosaccharides comparable to those found in breast milk.

In these preliminary tests, the oligosaccharides were used both as pre-treatment on the cells before infection and as treatment during infection in the presence of the virus.

From these partial pilot results, the concentration of HMOs appears to have a progressively incremental inhibiting effect from a concentration of 0.12mg/dL up to 05.mg/dL. Whereas at higher concentrations of 1 mg/dL, the inhibiting effect, although remaining lower than the control test values, appears to be reduced. In fact, a higher number of viral genome copies are found.

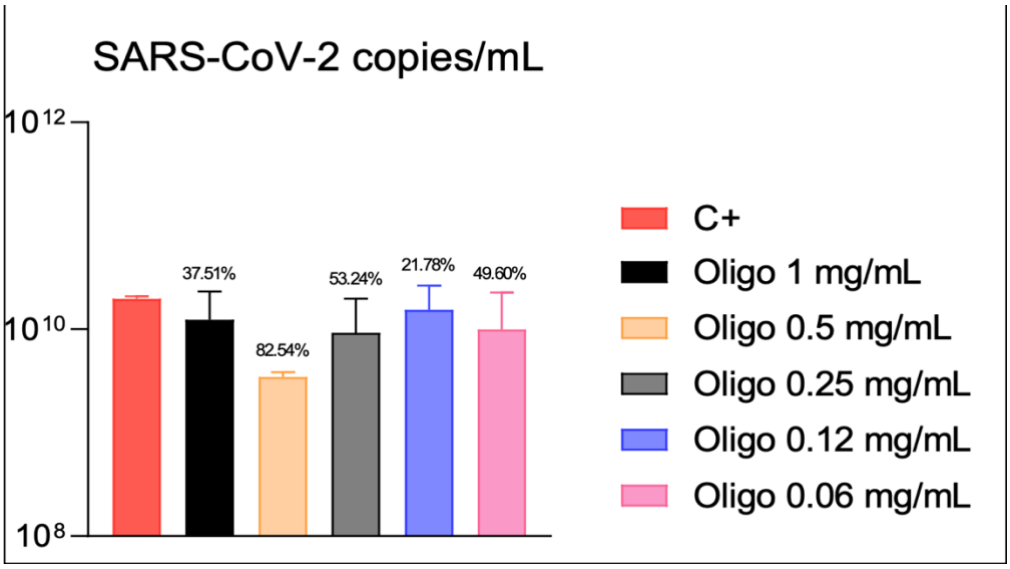

Figure 14. SARS-CoV-2 genome copies (copies/mL) in the supernatants of infected cells treated with different concentrations of a mixture of synthetic oligosaccharides. The inhibiting effect has an incremental value from a concentration of 0.12mg/dL up to 05.mg/dL but decreases at concentrations of 1mg/dL.

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Chapter 3

Discussion

The ability of milk from COVID-positive women to neutralise SARS-CoV-2 is confirmed by the study's findings. As already described in the literature, the percentage of neutralising samples from group A mothers at T0 was 100% and then decreased at the other time points (85% at T1 and 71% at T2). Additionally, a statistically significant progressive decrease from T0 to T2 is shown in the neutralising potency as expressed by NT50 in group A. The potential explanation for this phenomenon is that the mothers' COVID-19 infection status was confirmed at the moment of delivery, and thus temporally, at the time of colostrum sample collection (T0). Consequently, antibody levels in the milk may gradually decrease over time following infection; however, prior research has demonstrated the persistence of antibodies in breastmilk for up to six months and ninety days after infection.

When comparing the two different groups, it is evident that the numbers between group A and group B at timepoint 0 are different from each other and do not correspond. This limitation is due to the fact that colostrum collection, as is often the case in mothers, especially primiparas, is very difficult in the first few days of life, and the correct hand expression technique to obtain the colostrum sample is somewhat difficult for mothers. As a result, the number of samples in the two groups at these time points diverges, with 5 samples in Group A and 17 in Group B at T0, colostrum collection. Nevertheless, each scarcely obtained sample was analysed and presented in this article, in consideration of the considerable efforts made by mothers.

The most notable finding of this research validates what previous studies have suggested: samples obtained from negative mothers demonstrated neutralising potency. Antiviral activity was observed in the majority of negative samples, particularly at the second time point (41% at T0 versus 90% at T1). In fact, there was a statistically significant difference between the endpoint colostrum titre (NT50 1:11.3) and the titres seen at later time points, as determined by calculating NT50 (1:40 at T1 and 1:2.8 at T2). This difference was seen in the B group of mothers who did not have a previous infection.

In an attempt to reduce the bias of a possible previous misidentified infection or errors in group allocation, ELISA tests were conducted to determine the presence and quantification of antibodies directed against SARS-CoV-2.

Consistent with expectations, the mothers who tested positive for the virus in group A exhibited fluctuating levels and identities of anti-virus antibodies at different time intervals. As demonstrated by prior research, this presence eventually declines with time, albeit not substantially.

Unexpectedly, antibodies specific to SARS-CoV-2 have also been identified in the breast milk of negative mothers (group B), albeit to a diminished degree and with a lower IH% than in the breast milk of mothers in group A. The observed outcome may be attributed to prior asymptomatic SARS-CoV-2 infections that the mother failed to recognise and report. Alternatively, it may be influenced by the presence of antibodies, particularly those of the IgA class, that are designed against different coronaviruses but have the potential to cross-react with SARS-CoV-2 and thus disrupt the test, as demonstrated in prior research on plasma and saliva (Tsukinoki et al., 2021).

The antibodies identified in the breastmilk of positive mothers exhibit specific activity against SARS-CoV-2. This is further corroborated by the direct correlation observed between the presence of the antibodies and the neutralising activity of the breastfeeding samples. Conversely, no such correlation was observed in the milk of mothers who tested negative. This observation provides support for the hypothesis that the antiviral activity may be mediated by non-specific components that occur naturally in colostrum, operating independently of antibody activity (Morniroli et al., 2021).

Following this study, this study, preliminary tests were conducted to identify possible differences between the various samples in terms of compounds with known antiviral capacity. This finding would be of great importance in explaining, within the various samples of the same group, especially the samples of the negative mothers, group B, the described differences in terms of virus neutralising capacity. A first pilot analysis compared the concentrations of various subtypes of fatty acids, in particular LCPUFAs. Preliminary results showed a statistically significant correlation between the neutralising capacity of the sample and the eicosatrienoic acid content. Eicosatrienoic acid, commonly known as Mead acid (MA), is an omega-9 polyunsaturated fatty acid. Mead acid has a 20-carbon chain with three double bonds, located at positions 5, 8, and 11 from the methyl end of the fatty acid chain. Its chemical formula is C20H34O2 (Kawashima & Yoshizawa, 2023). It is synthesised in the body through elongation and desaturation of oleic acid, an omega-9 fatty acid. This process is catalysed by specific enzymes known as elongases and desaturases. Mead acid is found in significant quantities in various tissues, including brain and nerve tissues (Kawashima & Yoshizawa, 2023). It plays roles in multiple physiological functions within

the body. An increasing number of studies have reported the relationship between MA and diseases such as inflammation, cancer, dermatitis, and cystic fibrosis. Research suggests that mead acid may inhibit the growth of breast cancer cells, indicating potential anti-cancer properties (Kinoshita et al., 2014). Interestingly, mead acid appears to be involved in the viral infection process, particularly in the context of HCV and potentially HIV infections. Specifically, the synthesis of mead acid, regulated by the enzyme SCD (stearoyl-CoA desaturase), influences lipid peroxidation (LPO) and HCV replication. This suggests a direct link between mead acid metabolism and HCV infection (Yamane et al., 2022). Dysregulated fatty acid metabolism, including alterations in Mead acid levels, may be implicated in the progression of HIV infection (Ueland et al., 2023). However, ongoing research is necessary to fully comprehend the extent of Mead acid's role in viral infections and its therapeutic potential. The role of this omega-9 in SARS-CoV-2 infection has still been poorly explored.

Several other lipid compounds in breast milk have been investigated for their presumed antiviral capacity against SARS-CoV-2. In addition to long- and medium-chain fatty acids, and the monoglycerides derived from them, a category of lipid-derived molecules present in breast milk that has aroused great interest due to their antiviral capacity are the oxysterols. Among these, 25-hydroxycholesterol (25OHC) and 27-hydroxycholesterol (27OHC) are considered to participate in the innate immune response against viruses (Civra et al., 2019). Oxysterols, which are cholesterol derivatives formed through oxidation, have been shown to have antiviral properties, playing a protective role for infants during lactation. In particular, oxysterols derived from cholesterol oxidation have been identified as antiviral agents in human milk at various stages of lactation. In particular, 27-

hydroxycholesterol (27OHC), an oxysterol This oxysterol is a derivative of cholesterol obtained by endogenous oxidation, i.e., produced naturally by the human body. In a study by Marcello et al., 27OHC was added to in-vitro tests after inoculating susceptible cells with SARS-CoV-2, simulating a viral infection treatment protocol. 27OHC was able to effectively inhibit the replication of SARS-CoV-2 and another human coronavirus that causes the common cold (HCoV-OC43) without significant cytotoxicity at micromolar concentrations (Marcello et al., 2020). Although the quantum of oxysterols present in breast milk samples was not analysed in this study, a co-participating role in the viral inhibition displayed by the samples of seronegative mothers cannot be excluded.

As a follow-up of this study, a preliminary investigation was also conducted into the involvement of HMOs in the early stages of viral infection in negative women (group B). It is a fact that not all breast-feeding women generate the same types of oligosaccharides, an interesting fact to take into account. Their synthesis and composition from essential components depend on their fucosylation by the fucosyltransferases FUT2 (Secretor gene) and FUT3 (Lewis gene), which, like the FUT2- and FUT3-dependent blood group antigens, vary between mothers. Their fucosylation is regulated by the secretor and Lewis genes (Bode, 2020). It is possible that the great variety of neutralising activity found in the colostrum samples of the negative mothers in our sample is due to this inter-individual heterogeneity within the oligosaccharide pool.

Literature-based research indicates that human milk oligosaccharides (HMOs) employ a dual-pronged approach to protect against viral infections via their antiviral properties. To

begin with, HMOs obstruct viral entry by functioning as "decoy" receptors. They emulate the cell surface receptors that are typically targeted by viruses in order to gain entry. Through their interaction with these dummy receptors, HMOs efficiently obstruct the attachment and infiltration of viruses into host cells. The preliminary obstruction halts the pivotal stage of viral entry, thereby diminishing the likelihood of an effective infection (Moore et al., 2021). The aforementioned capability of HMOs is an essential barrier, fortifying the host's immunity against an extensive array of viral pathogens. HMOs inhibit viral replication and coating as a secondary mechanism of action, in addition to impeding virus entry. For replication and spread, viruses depend on the cellular apparatus of the host within infected cells. By impeding the coating of the virus and interfering with critical viral enzymes, HMOs disrupt this process. This interference effectively hinders the viral replication and dissemination, thereby halting the advancement of the infection (Donovan & Comstock, 2017; Moore et al., 2021). Furthermore, several studies propose that HOMs activate intracellular signalling mechanisms through their binding to the cell surface. This activation may result in the alteration of specific surface proteins, such as by modifying their dimerization, which subsequently prevents their receptors from binding to the virus. HMOs collectively establish themselves as a robust natural defence mechanism against viral infection, thereby providing neonates with an indispensable additional layer of protection via the act of lactation. In summary, human milk oligosaccharides employ a diverse array of mechanisms to repel viral infections. Initially, they function as decoy receptors, imitating receptors on the cell surface to impede virus entry, or they alter functional membrane receptors. Conversely, they impede the process of viral replication and encapsulation that

occurs within cells that are infected. According to a review by Moore et al., the scientific literature indicates that HMOs are capable of hinder macro viruses including influenza, rotavirus, respiratory syncytial virus, human immunodeficiency virus, and norovirus via glycan-binding interactions (Moore et al., 2021).

These preliminary results show that incremental concentrations of synthetic HMOs, comparable to those found in breast milk, correlate with an increasing percentage of inhibition. This seems to be true for concentrations between 0.12 mg/dl and 0.5 mg/dl. At higher concentrations of 1 mg/dl, however, the inhibitory effect is reversed, remaining lower than the control test. This trend shown by these pilot results, could be explained by the effect of oligosaccharides at the level of cellular membranes on viral entry. Indeed, while these findings are in their early stages and require multiple replications and productions, the observed behaviour of HMOs at higher concentrations appears to be indicative of their capacity to bind the virus. In fact, given that these are in vitro experiments, it is possible that a greater quantity of HMOs in the test well could 'concentrate' the density of SARS-CoV-2 viral particles, thereby maintaining them in close proximity to the cell surface. However, it is important to mention that this hypothesis considers the typical in vivo concentrations of HMOs in breast milk (5–15 g/L), which do not indicate this effect.

Other glycated and sialylated human milk components that have demonstrated antiviral action in previous studies were not explored for their antiviral effect in the present research. Mucins, for example, are glycated substances made up of very large molecules that have already been shown to have antiviral action via processes similar to those used by HMOs (Wedekind & Shenker, 2021).

These glycoproteins, which are mostly formed of carbohydrate chains known as glycans, are well known for their ability to defend against viral infections.

Mucins produce a thick, viscous gel-like network that acts as a physical barrier against viruses. This barrier successfully prevents viral attack and entry into host cells. According to research, mucins found in human milk, notably mucins 1 and 4, have antiviral capabilities against a variety of viruses, including HIV and the influenza virus. Their capacity to attach to viral particles, functioning as decoys and preventing them from interacting with host cells, is thought to be responsible for their antiviral activity (Lis-Kuberka & Orczyk-Pawiłowicz, 2019).

Mucins also have immunomodulatory properties. They have the ability to trigger immunological responses by activating certain immune cells. This activation results in the generation of immunological components that help the body fight viral infections. A number of studies have emphasised the significance of mucins in immunological activation, which has contributed to our knowledge of their antiviral action.

Mucins also help to produce a protective coating on mucosal surfaces, such as the respiratory and gastrointestinal systems. This layer works as a physical barrier between viruses and the body, preventing them from entering. This mucin-based protective barrier has been demonstrated in studies to be particularly efficient in preventing infection at mucosal entry points, which are typical sites of viral transmission (Dhar & McAuley, 2019).

Human milk mucins have also been studied for their ability to suppress particular viruses. Studies, for example, have looked at their anti-HIV efficacy. Although the capacity of breast

milk mucins to suppress HIV varies, there is evidence to indicate their potential as a natural defence against this virus (Mall et al., 2017).

The functional serum proteins found in breast milk are an additional compound recognised for its antiviral properties, which may account for the innate antiviral activity observed in the seronegative samples of the mothers in the present study.

Whey proteins demonstrate antiviral properties via a multitude of unique mechanisms. An action worthy of mention is their ability to hinder the attachment and entrance of viruses into host cells. Whey proteins have been found to interact with host cell receptors or viral genomes, thereby impeding virus entry, according to research. The virus is prevented from establishing infection in the host by this interference (Ng et al., 2015).

Furthermore, it has been noted that specific whey proteins can impede the replication processes of viruses. Certain milk proteins have been shown in studies to inhibit the replication of viruses, thereby preventing their multiplication and dissemination. This action impedes the advancement of the viral infection (Fan et al., 2020).

Lactoferrin, a significant whey protein, has garnered considerable interest due to its potent antiviral characteristics. It has been demonstrated to inhibit human immunodeficiency virus (HIV), herpes simplex virus, and hepatitis C virus, among others. Lactoferrin demonstrates its antiviral properties via a variety of mechanisms. One of these mechanisms functions by preventing viral particles from adhering to host cells through binding. Furthermore, lactoferrin has the capability to obstruct viral entry and disrupt viral replication processes (Campione et al., 2020).

Lactadherin, an additional significant whey protein that was previously mentioned, has exhibited potential in impeding viral infections. Research examining rotavirus, a prevalent gastrointestinal virus, unveiled that lactadherin attaches specifically to a number of variants of the virus, impeding their capacity to adhere to host cells. This action successfully restricts the capacity of the virus to establish an infection (Newburg et al., 1998).

Among the serum proteins in breast milk, tenascin C has also been shown in past years to have a powerful antiviral role. Its ability to inhibit viral infections has been particularly tested against the HIV virus (Fouda et al., 2013).

Previous studies have demonstrated that tenascin C, possesses a potent antiviral effect. Its ability to inhibit viral infections, particularly the HIV virus, has been evaluated.

Tenascin C is a glycoprotein that is thought to inhibits viral infections through a variety of mechanisms. According to research findings, a significant proportion of the neutralising activity of HIV-1 found in the milk of uninfected individuals is mediated by this protein (Mangan et al., 2019). This finding indicates that tenascin C is an essential factor in inhibiting the transmission of HIV-1 via breastfeeding. Additionally, tenascin C possesses antiviral activity across a broad spectrum. Furthermore, its antiviral properties in human milk, which extend beyond its impact on HIV-1, have been attributed to this substance. Research has demonstrated that tenascin C, which is found in breast milk, inhibits viral replication by preventing the assembly of viral particles. This interaction can occur via direct binding to viral particles, thereby impeding their capacity to cause infection within host cells (Zuliani-Alvarez & Piccinini, 2023).

In relation to COVID-19, recent research has shed light on its potential as a biomarker of disease severity in the context of other viral infections as well as COVID-19. The presence of this molecule in exosomes obtained from patients infected with COVID-19 suggests that it is implicated in the transmission of the virus, modulation of the immune system, and antiviral activity throughout the course of the infection.

It is essential to note, nevertheless, that tenascin-C has been regarded as a negative indicator of the severity of COVID-19 disease, and its transportation via exosomes has been attributed to a pro-inflammatory function, similar to other non-communicable human diseases like joint arthritis (Sur et al., 2021)

. As a result, in contrast to the literature regarding its activity against the HIV virus, the precise function of tenascin-C in SARS-CoV-2 infection remains unknown, whether it be positive or negative in terms of transmission and disease severity.

They whey fraction of human milk is also characterized by the presence of immunoglobulins, with secretory immunoglobulin A (sIgA) being a notable contributor to the antiviral defence mechanism as previously discussed in this dissertation. sIgA is essential for preventing viral infections on mucosal surfaces and is central to mucosal immunity (Fox et al., 2020). In order to accomplish this, it forms a complex with viruses, impeding their ability to adhere to host cells and thereby invalidating their pathogenic potential.

Notably, the antiviral mechanisms of whey proteins can differ in accordance with the particular virus in question. Various whey proteins are capable of selectively targeting distinct phases of the viral life cycle, encompassing attachment, entry, replication, and

release. Additionally, the synergistic effect produced by the combination of numerous whey proteins further enhances its antiviral potency (Hurley & Theil, 2011; Tsukinoki et al., 2021). As discussed at the beginning of this discussion, ELISA tests showed the presence of antibodies in the milk of seronegative women (group B). This result could be at least partly due to the known cross-reactivity of IgA, which is notably highly represented in colostrum, explaining the greater antiviral and neutralising power of this initial milk (Egwang et al., 2021).

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Chapter 4

Conclusions and future perspectives

The SARS-CoV-2 pandemic, in its dramatic scale, represented one of the great challenges for health research and innovation. On this occasion, as in the past, researchers all over the world joined forces to respond to the great worldwide need for cures, therapies, and finally, vaccines (Carvalho et al., 2023; Cucinotta & Vanelli, 2020).

This ominous occasion, however, led to great therapeutic and technological innovations. and validation of new treatment protocols and vaccine types (Niknam et al., 2022).

Some of the scientific research related to the pandemic also looked to the past in an attempt to seek new applications for new drugs, starting with already-known compounds. Breast milk certainly represents the largest and most powerful 'reservoir' of possible bioactive compounds with important functional properties.

The results of this study confirmed that breast milk, in addition to being a valuable source of 'passive' immunisation of the newborn, possesses broad-spectrum, innate antiviral properties inherent precisely in its complex, ever-changing, and yet perfect combination (Morniroli et al., 2021). To date, we still fail to fully capture not only the multitude of functional substances that are present in breast milk but also their precise mechanism of action. Indeed, the studies in the literature, some of which are cited in this dissertation, show only a fraction of the multiple functional and antiviral capabilities of human milk. Moreover, it is not yet possible to explore in detail what the mechanisms of action of these substances are. Mechanisms that we can, in fact, only surmise but that are likely to be

synergistic make it even more difficult to replicate in scientific studies the true magnitude of the effects of breast milk.

In relation to the results of this study, it is clear that further research is needed to identify the compounds, or the plurality of compounds, that are responsible for the remarkable results achieved. Future studies are necessary in order to identify, for example, by mass spectrometry, any molecular macro-differences between the samples analysed so as to have more information on the type of substances responsible for the antiviral action demonstrated. In view of what has been discussed and what is already in the literature, metabolomics, proteomics, or lipidomics studies in particular could also be useful in attempting to answer these questions (van Herwijnen et al., 2016; Yue et al., 2021). Secondly, it would be equally interesting to extend this type of test to other viral strains. This could be useful not only to verify that this antiviral effect of mother's milk is replicable on a wide range of pathogens but also to assess whether there are indeed differences in effect, for example, between viruses with and without envelopes. A possible difference in effect between viruses with and without envelopes, for example, would lead scientific research to focus on considering and testing substances and macromolecules capable of disrupting this peculiar viral structure, such as fatty acids, assuming at that point that this could be one of the mechanisms by which breast milk exerts its antiviral mechanisms (Thormar et al., 1987). It is worth remembering, however, how research has already confirmed that the substances responsible are certainly multiple and that the mechanisms of action are most likely synergistic. It certainly remains a fascinating field of research to recover this knowledge in order to apply it to the treatment and prevention of viral pathologies, in all age groups.

Scientific research over the last few decades has finally realised the potential of breast milk and has begun to look at it not only as the best food for infants but also as a powerful resource for possible therapies aimed at diseases both in infancy and at later stages of life. In this ever-expanding field of research, it is becoming increasingly evident that understanding the functional substances of breast milk, their characterization, and their mechanisms is a valuable potential for new therapies. Efforts to harness the potential of mother's milk, which has built up and changed in tandem with the history, genome, and diseases of the human race, will certainly lead to the 're-discovery' of substances that evolution has modelled over the millennia for the benefit of human health.

In conclusion, breast milk is the evolutionary footprint that has allowed us to thrive as a species, adapting to every adverse condition. And perhaps it is in this powerful tool, perfected over millennia, that we must look to find the answers to future challenges.

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