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Antiviral properties of whey proteins and their activity against SARS-CoV-2 infection

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ABSTRACT

Native and chemically modified whey proteins and their peptide derivatives are encountering the interest of nutraceutical and pharmaceutical industries, due to the numerous properties, ranging from antimicrobial to immunological and antitumorigenic, that result in the possibility to employ milk and its protein components in a wide range of treatment and prevention strategies. Importantly, whey proteins were found to exert antiviral actions against different enveloped and non-enveloped viruses. Recently, the scientific community is focusing on these proteins, especially lactoferrin, since in vitro studies have demonstrated that they exert an important antiviral activity also against SARS-CoV-2. Up-to date, several studies are investigating the efficacy of lactoferrin and other whey proteins in vivo. Aim of this review is to shed light on the most relevant findings concerning the antiviral properties of whey proteins and their potential applications in human health, focussing on their application in prevention and treatment of SARS-CoV-2 infection.

1. Introduction

This review has been carried out with the aim of summarizing the state-of-the-art on the role of lactoferrin and whey proteins in SARS-CoV-2 infection and their potentiality in COVID-19 treatment. A preliminary literature search was carried out on Medline (PubMed). Inclusion criteria were: In silico, In vitro and in vivo studies, clinical trials. Following, a more comprehensive literature search was carried out on Medline (Pubmed), EMBASE (Elsevier), Google Scholar. Additional papers were added from the bibliography of the most relevant articles.

2. Whey proteins

Milk contains numerous bioactive components including proteins, lipids and oligosaccharides fulfilling several and pleiotropic functions that are physiologically directed to promote growth (Richter et al., 2019) and a healthy development of new-borns and children (Eriksen et al., 2018; Hill & Newburg, 2015; Kim & Yi, 2020; Mosca & Giannì, 2017). However, milk continues to be a basic food also for adults, due to other properties (e.g., antimicrobial, immunological and antitumorigenic), which can be more important than the nutritional function (Clare et al., 2003; Gill & Cross, 2000; Kim et al., 2020). Indeed, most of the studies in the nutraceutical field are focusing on milk components and their derivatives in the attempt to exploit milk bioactive molecules for diseases' treatment and prevention, and to find new accessible and health compatible drugs or to improve conventional therapies (Davies et al., 2018; Dybdahl et al., 2021; Galley & Besner, 2020; Kennedy et al., 1995; Sánchez et al., 2021).

Many of the biological and functional properties of milk are due to milk proteins (Donovan, 2019; Haschke et al., 2016; Li et al., 2017; Lönnerdal, 2003; Zhu & Dingess, 2019). Beside caseins, whey proteins represent one of the two major fractions of milk proteins (Yamada et al., 2002). Milk also contains a third class of proteins known as mucins present in the fat globule membrane (Lönnerdal et al., 2017).

Whey proteins constitute approximately 20% of milk proteins but their absolute content and ratio to caseins varies greatly, depending on the species but also on the lactation stage, being more abundant in colostrum than in mature milk (Atkinson & Lönnerdal, 1995; Lemay et al., 2013).

Whey proteins mostly represented in human milk include α-lactalbumin, immunoglobulins and serum albumin, while a minor fraction is represented by lactoferrin, glycomacropeptide, lactoperoxidase and lysozyme (Donovan, 2019; Haschke et al., 2016). Bovine milk has a similar composition except for the presence of a high amount of

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β-lactoglobulin that is completely absent in human milk. Bovine milk also contains less α-lactalbumin than human milk (Schack et al., 2009).

Whey proteins are considered to have the highest nutrition value due their content of essential amino acids. Besides this, whey proteins exert numerous biological and functional activities that influence different biological processes promoting bone growth and muscle strength, lowering cholesterol, improving cognitive functions, and regulating mood. In addition, they also display anti-oxidative, anticancer, antimicrobial, anti-inflammatory and immunomodulatory functions (Akhavan et al., 2014; Krissansen, 2007; Layman et al., 2018; Morniroli et al., 2021; Teixeira et al., 2019).

3. Antiviral activity of whey proteins

Due to their health-enhancing properties, bioavailability and safety, both native or chemically modified whey proteins and peptide derivatives are studied for their potential pharmacological activity alone or in synergy with other drugs against several disease including virus infections (Małaczewska et al., 2019) (see Table 1). It has been reported that human casein exerts an antiviral activity towards Human immunodeficiency virus (Berkhout et al., 2002), Human hepatitis B virus (Hara et al., 2002) and Human rotavirus (Inagaki et al., 2014). Despite this, most of the antiviral properties of milk are attributed to whey proteins that are known to exert important antiviral actions against different enveloped and non-enveloped viruses (Wakabayashi et al., 2014) including influenza virus A (H1N1), human cytomegalovirus, human immunodeficiency virus (HIV1), hepatitis B and C virus (Florian et al., 2013; Liao et al., 2012; Redwan et al., 2014), avian influenza A (H5N1) (Taha et al., 2010), herpes simplex virus type 1 and 2, hantavirus, poliovirus, influenza virus A (A1N1) (Sitohy et al., 2010a; Sitohy, Besse, Billaudel, Haertlé, & Chobert, 2010b), human rotavirus, human papilloma virus and enterovirus (Ng et al., 2015). The most important antiviral properties have been ascribed to native lactoferrin and peptide derivatives such as lactoferricin and lactoferrampin (Kell et al., 2020; Valenti & Antonini, 2005; Van der Strate et al., 2001; Wakabayashi et al., 2014; Giansanti et al., 2016), that, in some cases, showed strongly augmented antiviral effects compared to the native protein (Shestakov et al., 2012). Moreover, numerous studies showed that native lysozyme and chemically modified β-lactoglobulin, α-lactalbumin and some of their hydroxylated peptides, from human and other mammalians, also display valuable antiviral effects (Oevermann et al., 2003). Chemical modifications, such as acetylation or the addition of 3-hydroxyphthaloyl acid, enhance the antiviral properties of these proteins, presumably leading to charges change and redistribution (Pan et al., 2006). Indeed, kinetic studies demonstrated that the presence of negative charges strongly increases the affinity of whey proteins for viral cell target receptors and for viral proteins (Zeder-Lutz et al., 1999).

Despite native lactoferrin as well as other whey proteins, including lysozyme and chemically modified β-lactoglobulin and α-lactalbumin, have shown antiviral activity towards a greatly overlapping spectrum of pathogens they exert their antiviral activity with several, even if not fully elucidated, mechanisms of action (Ng et al., 2015). Most of the known mechanisms of actions involve interactions of whey proteins with host cell receptors or with the viral genomes preventing viral entry and replication in cells. Studies performed on human deficiency virus 1 (HIV-1) demonstrated that bovine lactoferrin and its hydrolysis peptide lactoferricin inhibit virus entry by acting on CXCR4 and CCR5 receptors (Berkhout et al., 2004), and the apo form of bovine lactoferrin was demonstrated to have a role in the inhibition of HIV-1 replication (Puddu et al., 1998). On the other hand, the inhibition of HIV-1 entry in CD4 cells by modified β-lactoglobulin and α-lactalbumin was showed to be mainly due to interactions with the gp120 envelope protein (Neurath, Debnath, Strick, Li, Lin, & Jiang, 1995). In human cytomegalovirus infection, lactoferrin, methylated β-lactoglobulin and α-lactalbumin mainly inhibit virus replication and transcription by interacting with the viral genome (Chobert et al., 2007; Swart et al., 1998). Similar mechanisms of action were shown in studies carried out on hepatitis B and C viruses and on herpes simplex virus type 1 and 2, where inhibition by human and bovine lactoferrin and peptide derivatives and by α-lactalbumin, β-lactoglobulin and lysozyme, respectively, depends by interaction between viral and cell proteins that interfere with virus entry and multiplication (Sitohy et al., 2007). It was further demonstrated that lactoferrin acts also interacting with heparan sulfate proteoglycans

Table 1
Antiviral activity of the main whey proteins.

Whey Protein	Virus	References
Lactoferrin	Friend Virus Complex (mouse virus)	Lu et al., 1991
	Herpes simplex virus type 1and 2	Marchetti et al., 1998
	Hepatitis C virus	Ikeda et al., 2000
	Human rotavirus	Superti et al., 2001
	Hantavirus	Murphy et al., 2001
	Adenovirus	Arnold et al., 2002
	Feline Calicivirus (feline virus)	McCann et al., 2003
	Poliovirus	Seganti et al., 2004
	Human immunodeficiency Virus	Berkhout et al., 2004
	Human cytomegalovirus	Beljaars et al., 2004
	Sindbis virus and semliki forest virus	Waarts et al., 2005
	Human papillomavirus	Mistry et al., 2007
	Human echovirus	Ammendolia et al., 2007
	Japanese Encephalitis Virus	Chien et al., 2008
	Hepatitis B virus	Li et al., 2009
	Enterovirus	Yen et al., 2011
	Respiratory syncytial virus	Gualdi et al., 2013
	Influenza virus A (H1N1)	Superti et al., 2019
Angiogenin	Human immunodeficiency Virus	Wang et al., 2000
Milk mucin	Human rotavirus	Yolken et al., 1992
	Poxvirus	Habte et al., 2007
	Human immunodeficiency Virus	Mall et al., 2017
β-lactoglobulin (Native or chemically modified)	Human immunodeficiency Virus	Neurath et al., 1996
	Influenza virus A (H1N1)	Schoen, Corver, Meijer, Wilschut, & Swart, 1997; Sitohy et al., 2010a
	Herpes simplex virus type 1and 2	Neurath et al., 1998
	Human cytomegalovirus	Chobert et al., 2007
	Human papillomavirus	Taha et al., 2010
	Avian influenza A (H5N1)	Lu et al., 2013
	Human rotavirus	Ng et al., 2015
α-lactalbumin	Herpes simplex virus type 1and	Oevermann et al., 2003
	Human immunodeficiency Virus	Marshall, 2004
	Human cytomegalovirus 2	Chobert et al., 2007
Lysozyme	Herpes simplex virus type 1	Oevermann et al., 2003
	Human immunodeficiency Virus	Behbahani et al., 2018
Lactadherin	Human rotavirus	Bojsen et al., 2007
Tenascin-C	Human immunodeficiency virus	Mangan et al., 2019

(HSPs) on the host cell surface as reported in in vitro studies carried out on alphavirus infection (Waarts et al., 2005).

In addition, the antiviral action of whey proteins was also shown to depend by other mechanisms including the inhibition of viral shedding, as reported in in vitro studies concerning the effects of lactoferrin on Hantavirus infection, and cytopathic effects, as reported by studies regarding lactoferrin and lactoperoxidase's action on influenza virus A (H1N1) and human echovirus (Pietrantoni et al., 2006; Shin et al., 2005). Another mechanism of action was observed in a study carried out on infuenza A virus. This work demonstrated that the interaction between bovine lactoferrin and viral haemagglutinin leads to the inhibition of the virus-induced haemagglutination and consequentially to a reduction of infection (Ammendolia et al., 2016); a similar mechanism of action was also observed in previous studies on influenza virus A and glycomacropeptide (Kawasaki et al., 1993).

Although almost all of the reported and known antiviral mechanisms of actions involve a direct effect on virus entry and replication caused by protein/virus interaction, greatly influenced by proteins' charge distribution, recent studies carried out on lactoferrin and norovirus showed a possible, but not yet fully understood, indirect mechanism of action in reducing norovirus infection involving a lactoferrin-mediated induction of innate interferon response (Oda et al., 2021).

Following the coronavirus emergency, several researchers started to investigate molecules able to adjuvate and implement current conventional therapies for prevention and treatment of Covid-19. Several of these studies focused on milk and whey proteins, especially lactoferrin (see Table 2). This article aims to provide an updated review on the antiviral properties of whey proteins and their peptide derivatives examining the most recent findings upon anti-SARS-CoV-2 effects and health applications.

4. Anti SARS-Cov-2 activity of whey proteins

Fan and co-workers demonstrated for the first time that whey proteins from human breast milk and from other species, including goat and cow milk, inhibit both SARS-CoV-2 and pangolin coronavirus (GX_P2V) by blocking virus entry and replication in Vero E6 and A549 cell lines, with a EC_{50} of about 0.13 and 0.5 mg/ml of total protein, respectively; a similar effect was also demonstrated for some commercial bovine milk formula (Fan et al., 2020). The authors also suggested that breast milk inhibits both virus entry and replication presumably by reducing the affinity between SARS-CoV-2 S protein and ACE-2 and by interfering with viral RNA-dependent RNA-polymerase, respectively. This study also verified the contribution of lactoferrin in these processes. Both recombinant human and bovine lactoferrin showed a valuable but lower viral inhibition compared to the whole whey protein, suggesting that also other whey proteins could be involved in reducing infectivity, presumably with a synergic mechanism. Considering Lf concentration in breast milk during SARS-CoV-2 infection, no difference was found between SARS-CoV-2 positive mothers and controls. However, in a specific subgroup, symptomatic mothers displayed lower breast milk Lf concentrations as compared to asymptomatic mothers and healthy controls, suggesting that SARS-CoV-2 infection could cause variations in the breast milk concentration of Lf. (Briana et al., 2021). Considering the individual whey proteins, in addition to lactoferrin, other whey proteins including beta-lactoglobulin and lysozyme were analysed for their potential antiviral activity against SARS-CoV-2 (Pradeep et al., 2021) and in reducing inflammation, infiltration, and activation of innate immune cells such as neutrophils and macrophages (Mann & Ndung'u, 2020) (Table 2).

4.1. Lactoferrin

Previous studies already demonstrated the antiviral action of lactoferrin against SARS-COV pseudovirus performed by inhibiting virus entry with a mechanism that involves binding to heparan sulfate (HS)

Table 2
Anti-Coronavirus activity of whey proteins.

Protein	Type of experiment	Notes	References
Whey proteins	In vitro	The whole human breast milk whey proteins association is active towards SARS-CoV-2 and pangolin coronavirus	Fan et al., 2020
Lactoferrin (Lf)	In silico	Lf displays high affinity with the spike CTD1 domain	Campione et al., 2021
	In silico	Lf binds to sialic acid sheltering the cell from the virus attachment	Miotto et al., 2021
	In silico	Lf competes with spike protein for binding to ACE2 receptor	Miotto et al., 2021.
	In silico	Milk peptides are multitargeted anti-COVID-19 drug candidates	Pradeep et al., 2021
	In vitro	Lf hinders SARS pseudovirus binding at the level of heparan sulfate proteoglycans	Lang et al., 2011
	In vitro hypothesis	Lf reduces SARS-CoV-2 infectivity inhibiting cathepsin L activity	Madadlou, 2020
	In vitro hypothesis	Lf blocks spike protein furin-cleavage site	Naidu et al., 2020
	In vitro	Lf blocks SARS-CoV-2 entry by interaction with heparan sulfate	de Carvalho et al., 2020 (preprint)
	In vitro	Lf alone is less active than the whole human breast milk whey proteins association	Fan et al., 2020
	In vitro	Lf prevents host attachment of SARS-Cov-2 and other coronavirus through multiple interactions with cell membrane heparan sulfate proteoglycans	Hu et al., 2021
	In vitro	Lf blocks SARS-CoV-2 virus attachment to cellular heparan sulfate and enhances of interferon responses	Mirabelli et al., 2021
	In vitro	Lf acts also as an immune modulator of the antiviral immune response	Salaris et al., 2021
	In vitro	Lf potentiates the effect of remdesivir towards SARS-CoV-2	Mirabelli et al., 2021; Hu et al., 2021
	In vitro	Lf enhances hypothiocyanite anion ($OSCN^-$) activity towards SARS-CoV-2	Cegolon et al., 2021
	In vivo	Oral and intranasal liposomal Lf causes faster clinical recovery	Campione et al., 2020a
	In vivo hypothesis	Lf could be used as single- or combination treatment for both prevention and therapy of COVID-19	Chang et al., 2020
	In vivo	Combined oral administration of liposomal Lf and zinc solution allowed prompter recovery of patients	Cegolon & Mastrangelo, 2020a; 2020b

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Table 2 (*continued*)

Protein	Type of experiment	Notes	References
	In vivo	No definitive conclusion about Lf potential benefit as a support therapy	Algahtani et al., 2021
	In vivo	Milk Lf levels are potentially influenced by the severity of maternal COVID-19 infection during pregnancy.	Briana et al., 2021
	Retrospective study	Lf supplementary treatment in counteracting SARS-CoV-2	Rosa et al., 2021
	In vivo hypothesis	Lf could display high therapeutic value against COVD-19 due to its iron-chelating activity	Habib et al., 2021
Lysozyme	In vitro and in vivo (mice)	Inhalable composite niclosamide-lysozyme particles active towards SARS-CoV-2	Brunaugh et al., 2021
	In vivo hypothesis	Potential beneficial role of lysozyme presents in tears on limiting hypothetical ocular surface transmission of SARS-CoV-2	de Freitas Santoro et al., 2021
Beta-lactoglobulin	In silico	Inhibition of cathepsin L and possible inhibition of SARS-CoV-2 entry	Madadlou, 2020
	In silico	possible binding between beta-lactoglobulin derived peptides and spike protein	Çakır et al., 2021.
Lactoperoxidase	In vitro	Generates $OSCN^-$ that is effective to inhibit several viruses	Cegolon & Mastrangelo, 2020b, Cegolon et al., 2021

(Lang et al., 2011). The study, carried out on SARS pseudovirus infected HEK293E/ACE2-Myc cells, demonstrated that lactoferrin binds on the cell surface mainly at the level of heparan sulfate proteoglycans, hindering binding of the spike protein to the cell surface in an ACE-2 independent mechanism (Lang et al., 2011). SASR-CoV-2 entry in target cells mainly involves the interaction of the viral spike glycoprotein with its cognate cellular receptor angiotensin-converting enzyme 2 (ACE2) (Hoffmann et al., 2020). However, the interaction of the virus with other cell molecules such as heparan sulfate, that mediates the virion adhesion to the cell membrane, facilitating the interaction with ACE-2, and with cell proteases, including lysosome-localized Cathepsin L and serine proteases of the TMPRSS family, that favour internalization, is also required for SARS-CoV-2 entry (Zang et al., 2020; Zhang et al., 2020; Shang et al., 2020). Indeed, in silica studies, suggested that lactoferrin directly interacts and binds to sialic acid, sheltering the cell from the virus attachment; moreover, a possible competition between lactoferrin and ACE2 for binding to the spike protein was also postulated (Miotto et al., 2021). Recently, Hu et al. (2021), demonstrated that both bovine and human lactoferrin efficiently inhibit the entry of SARS-CoV-2 pseudovirus and other common coronaviruses, HCoV-OC43, HCoV-NL63, and HCoV-229E, in several cell lines including Vero E6, Calu-3 and 293 T-ACE2 cells by preventing host attachment through multiple interactions with the negatively charged cell membrane HSPGs. In particular, the inhibition mediated by bovine lactoferrin was higher than that obtained with human lactoferrin, with EC50 values for HCoV-OC43, HCoV-NL63, and HCoV-229E viruses from 11.2 to 37.9 μg/ml (bovine lactoferrin) and from 35.7 to 117.9 μg/ml (human lactoferrin), while, for SARS-CoV-2 the EC50 value of bovine lactoferrin was around 500 μg/ml. Furthermore, in Vero E6 cell bovine lactoferrin also inhibited SARS-CoV-2 replication and virion production, but only when lactoferrin was added before the virus entry. The higher antiviral activity exerted by bovine lactoferrin was also demonstrated to be due to its greater HS binding affinity (Hu et al., 2021).

Bovine lactoferrin was demonstrated to be effective in reducing in vitro progeny virus yield of up to 84% in Vero E6 and 68% in A549 cell lines at a concentration of 1 mg/ml. In addition, the inhibitory activity of lactoferrin on SARS-CoV-2 was attributed to the ability to block the virus entry interacting with HS (de Carvalho et al., 2020). However, other authors reported that the antiviral effect of lactoferrin, besides being dose-dependent, greatly varies depending on the experimental conditions (e.g., being evident only after pre-infection treatment) and cell type (Campione et. al., 2020a). Indeed, it is important to note that in a study carried out on iAEC2s and Huh7 cells the anti-SARS-CoV-2 effects of lactoferrin were demonstrated within a nanomolar range (Mirabelli et al., 2021).

Despite the fact that there are no experimental evidences that lactoferrin-mediated inhibition of cathepsin L can affect SARS-CoV-2 internalization, other molecules with similar inhibitory properties on cathepsin L, such as K77, were already identified as able to strongly affect SARS-CoV-2 infection in different cell lines (Mellott et al., 2021), suggesting a potential role of lactoferrin in reducing SARS-CoV-2 viral infectivity through the inhibition of cathepsin L activity (Madadlou, 2020), considering that inhibitors of cathepsin L have been demonstrated to prevent severe acute respiratory syndrome coronavirus entry (Simmons et al., 2005). In addition, at the junction of S1-S2 subunits of spike proteins is present a polybasic cleavage site, required for the cellular virus uptake, that is processed by furin and other cell proteases (Andersen et al., 2020). In this context, highly basic proteins, such as lactoferrin, might compete for the spike protein furin-cleavage site and inhibit virus entry (Naidu et al., 2020).

In addition to the inhibition of virus entry and/or virus replication, several in vitro studies reported that lactoferrin acts against SARS-CoV-2 infection enhancing the antiviral host cell response. Modulation of host cell immunity was identified as another main and indirect mechanism of action related to the anti-SARS-CoV-2 effect of lactoferrin, exerted by the induction of an increased expression of interferon and interferon-stimulated genes (Mirabelli et al., 2021). Indeed, lactoferrin was demonstrated to significantly induce the expression of interferon and anti-inflammatory and pro-inflammatory cytokines in uninfected caco-2 cells inducing IFNA1, IFNB1, TLR3, TLR7, IRF3, IRF7 and MAVS genes and enhancing the antiviral immune response. It was also showed that the expression of RNA-dependent RNA polymerase (RdRp) and E gene (CoVE) was significantly reduced in lactoferrin-treated SARS-CoV-2 infected Caco-2 cells (Salaris et al., 2021). Moreover, this study confirmed previous findings in which lactoferrin was also shown to decrease the massive production of inflammatory cytokines, including IL-6 (Cutone et al., 2014; Zimecki at al., 2021). Besides these two main mechanisms of action, lactoferrin was reported to be an effective inhibitor of cysteine proteases, including cathepsin L (Ohashi et al., 2003), a key enzyme in virus internalization, involved in spike protein processing (Ou et al., 2020).

Importantly, in the attempt to find new possibly stronger anti-SARS-CoV-2 treatments, an increasing number of in vitro studies demonstrated synergies between lactoferrin and other antiviral molecules, including several FDA-approved drugs. Bovine lactoferrin was demonstrated to display a synergistic antiviral effect with remdesivir, an FDA-approved antiviral drug which inhibits SARSCoV-2 polymerase, potentiating of about 8-fold its efficacy (Hu et al., 2021; Mirabelli et al., 2021). Lactoferrin was also reported to enhance the antiviral activity of the hypothiocyanite anion ($OSCN^-$) against SARS-CoV-2 in combination treatments performed on Vero E6 and HEK293T cell lines (Cegolon et al., 2021).

Findings concerning the possible molecular mechanisms behind anti-SARS-CoV-2 activity of lactoferrin were also supported and complemented by several in silico studies. Using a protocol based on the 2D Zernike Polynomials, Miotto el al. (2021) carried out a computational study to evaluate the possibility of lactoferrin to bind or interact with several cell and virus substrates considered as involved in SARS-CoV-2 infection. Results found for sialic acid, heparan sulfate, ACE2, spike protein, and other membrane and envelope proteins, highlighting a possible competition between ACE2 and lactoferrin for binding of SARS-CoV-2 spike protein (Miotto et al., 2021). Other molecular docking simulations confirmed the high affinity of bovine lactoferrin to the spike CTD1 domain, which is the same region of the spike protein that is involved in the binding with ACE-2, and a possible but significantly lower affinity of human lactoferrin (Campione et al., 2021), opening the possibility that lactoferrin could exert its blocking activity on virus entry also hampering spike protein for the binding to its cognate receptor ACE-2. Lactoferrin was also investigated for its function of iron binding protein. Dalamaga et al. (2020) reviewed the role of iron in Covid-19 inflammation and on the potential role of iron chelator agents in reducing the SARS-CoV-2 inflammation related to iron overload. Similarly, Habib et al. (2021) hypothesised a high therapeutic value of lactoferrin for its iron-chelating activity.

Considering the results obtained in vitro and in silico studies, numerous clinical trials are in progress to investigate the anti-SARS-CoV-2 action of lactoferrin in vivo, where lactoferrin could be used as single- or combination treatment for both prevention and therapy of COVID-19 disease (Chang et al., 2020). To date there is still little evidence coming from clinical studies; however, a prospective observational study on 75 COVID-19 positive patients demonstrated that the combined oral administration of liposomal lactoferrin and zinc solution for 10 days allowed a complete and prompter recovery of all treated patients compared to untreated controls within the first 5 days of treatment (Cegolon et al., 2020a). Moreover, a similar treatment (liposomal Lf + Zinc) was effective in preventing the disease in healthy people that were exposed to the virus (Serrano et al., 2020). In addition, another promising ongoing clinical trial is studying the effect of both local treatment with liposomal formulation of lactoferrin, administered as intra-nasal spray, and oral assumption in the treatment of coronavirus infection and inflammation and in preventing a severe disease progression in asymptomatic or mild patients (Campione et al., 2020b). In a small retrospective study on asymptomatic, paucisymptomatic, and moderate symptomatic COVID-19 patients, the time required to achieve SARS-CoV-2 RNA negativization in patients orally treated with bovine Lf was significantly lower compared to that observed in Lf-untreated ones (Rosa et al., 2021). However, another study showed that the differences regarding reduction in symptoms and laboratory indices between patients receiving approved Egyptian COVID-19 management protocol and patients receiving the same treatment plus lactoferrin were not statistically significant. Further studies with larger samples as well as longer-term trials to understand the role of Lf in treating SARS-CoV-2 are required (Algahtani et al., 2021; Wang et al. 2020).

4.2. Lysozyme

Antiviral properties of lysozyme are mainly attributed to its cationic nature that allows it to bind negatively charged membrane molecules (Małaczewska et al., 2019; Sava, 1996). Lysozyme resulted effective against herpes simplex and herpes zoster (Ferrari et al., 1959) and able to affect HIV-1 replication (Lee-Huang et al., 1999). Even if there are still not studies that demonstrate the efficacy of lysozyme in the inhibition of SARS-CoV-2, recently de Freitas Santoro and co-workers discussed the potential beneficial role of lysozyme found in tears in limiting hypothetical ocular surface transmission of COVID-19 (de Freitas Santoro et al., 2021).

Beside its antiviral action, in the context of lung diseases, there is strong evidence that lysozyme aerosol treatment is effective in reducing inflammation and lung tissue injuries in animal models of pneumonia and emphysema (Cantor et al., 2002; Griswold et al., 2014). Furthermore, lysozyme exerts neuroprotective functions (Helmfors et al., 2015) that could be helpful to prevent neurological COVID-19 outcomes. In vitro studies on SARS-CoV-2 infected Vero E6 cells demonstrated that lysozyme enhances the antiviral action of niclosamide. Interestingly, lysozyme was also found to significantly reduce lung viral load in SARS-CoV-infected mice after intranasal administration in combination with niclosamide and to exert an immunomodulatory action (Brunaugh et al., 2021).

4.3. Beta-lactoglobulin

Beta-lactoglobulin is a whey protein belonging to lipocalin family; it is very abundant in cow and goat milk but is absent in human milk. Several studies report the antiviral properties of chemically modified 3-hydroxyphthalic anhydride- bovine beta-lactoglobulin showing its potent inhibition activity against HIV, HSV-1, HSV-2, (Neurath et al., 1995, 1998) and several HPVs including HPV6, HPV16 and HPV18 (Lu et al., 2013). These results elicit the possible action of chemically modified beta-lactoglobulin against SARS-CoV-2. Although there are still no in vitro studies in this regard, peptides derived from the hydrolysis of goat milk beta-lactoglobulin were studied for their anti-SARS-CoV-2 activity using in silico approaches. In particular, Beta-lactoglobulin peptides were analysed to investigate their effectiveness against SARS-CoV-2 proteases and spike protein and their inhibitory activity on ACE and DPP-4 and furin enzymes was displayed, based on BIOPEP calculations. Docking studies also demonstrated a possible binding between beta-lactoglobulin derived peptides and spike protein, suggesting their potential role in inhibiting SARS-CoV-2 infection (Çakır et al., 2021). Similarly, to what previously discussed for lactoferrin, beta-lactoglobulin was demonstrated to inhibit cathepsin L, suggesting its potential antiviral role in SARS-CoV-2 inhibition of virus entry (Madadlou, 2020). In addition to its antiviral activity, beta-lactoglobulin was demonstrated to effectively enhance immune responses promoting cell proliferation (Tai et al., 2016). Overall, these findings suggest that beta-lactoglobulin is a promising molecule that needs to be further investigated as an adjuvant in COVID-19 treatment.

4.4. Lactoperoxidase

Lactoperoxidase is a whey protein belonging to the heme peroxidase family. In the presence of hydrogen peroxide, lactoperoxidase reacts with thiocyanate (SCN^-) generating the antimicrobial hypothiocyanite anion ($OSCN^-$). This activity counts for its strong antimicrobial activity, also established against several viruses such as HIV, HSV-1, adenovirus, echovirus, respiratory syncytial virus (RSV) and influenza virus (Cegolon & Mastrangelo, 2020b). Interestingly, in vitro studies on cell models demonstrated that $OSCN^-$ is effective in inhibiting SARS-CoV-2 infection at a micromolar range (Cegolon et al., 2021), suggesting that lactoperoxidase activity towards SARS-CoV-2 should be further investigated.

5. Conclusions and perspectives

Whey proteins and their biologically active peptides encountered great scientific interest as nutraceuticals in the prevention and treatment of several viral diseases, due to their important antiviral and anti-inflammatory properties and to their wide availability and biosafety.

The ongoing Covid-19 pandemic disease is still now lacking effective treatments and the individuation of new molecular targets and low-toxicity antiviral drugs can be currently an important weapon to fight with. This emergency led scientists to investigate the anti-SARS-CoV-2 properties of milk and whey proteins. Several in vitro studies carried out on different cell types demonstrated a strong antiviral and anti-inflammatory activity of whey proteins, especially lactoferrin, against

SARS-CoV-2 and a possible role in reducing pulmonary injuries and other detrimental COVID-19 outcomes. Furthermore, in silico studies of molecular docking gave strong evidence of putative binding sites on both cellular and viral molecules involved in virus infection, helping in the discovery of antiviral molecular targets. These results suggested that lactoferrin together with other whey proteins including beta-lactoglobulin, lysozyme and lactoperoxidase could be excellent candidates as new antiviral and immunomodulatory molecules (Mann & Ndung'u, 2020). Importantly, lactoferrin was also found to enhance the antiviral activity of some existing anti-COVID-19 drug when used in combination treatments, eliciting the possibility that it could be used both alone or in combination, to help conventional therapies in inhibiting infection and reducing inflammation. Indeed, an increasing number of ongoing clinical studies are supporting in vitro results, confirming the possible usefulness of lactoferrin and whey proteins in COVID-19 prevention and treatment.

Several "in silico" and "in vitro" studies indicate that lactoferrin and whey proteins are promising therapeutic agents against SARS-CoV-2 infection. However, from the surveyed literature, several points should be better addressed (larger cohorts of patients, pharmaceutical formulation and co-treatments, dosage and duration of treatments) to assess the effectiveness of lactoferrin and whey proteins in targeting COVID-19 infection and their use as new drug candidates against SARS-CoV-2.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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