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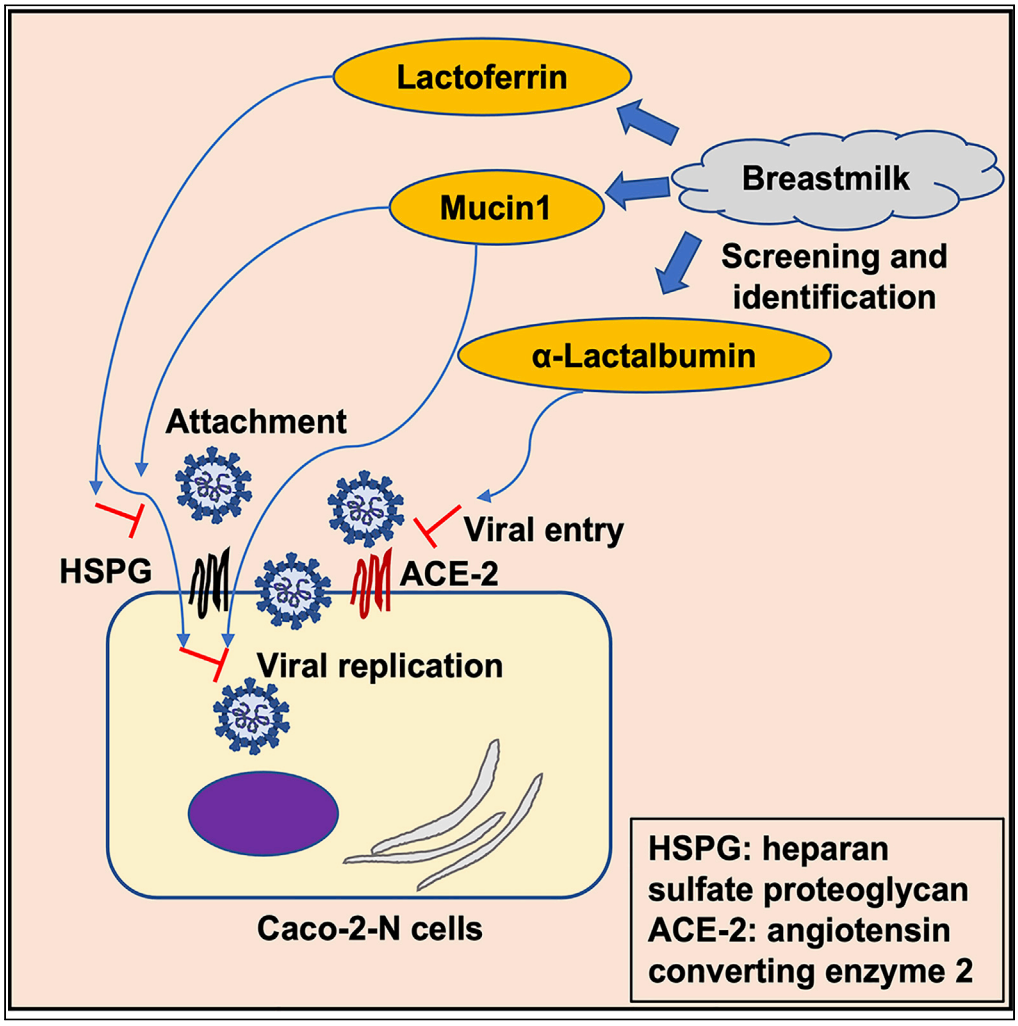

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Highlights

Lactoferrin (LF), MUC1, and α-lactalbumin (α-LA) suppressed SARS-CoV-2 infection

LF and MUC1 inhibited viral attachment, entry, and postentry replication

LF and MUC1 impair SARS-CoV-2 to attach heparan sulfate proteoglycan

MUC1, LF, and α-LA could also suppress SARS-CoV-2 variants infection

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Identified human breast milk compositions effectively inhibit SARS-CoV-2 and variants infection and replication

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SUMMARY

The global pandemic of COVID-19 caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection confers great threat to public health. Human breast milk is a complex nutritional composition to nourish infants and protect them from different kinds of infectious diseases including COVID-19. Here, we identified that lactoferrin (LF), mucin1 (MUC1), and α-lactalbumin (α-LA) from human breast milk inhibit SARS-CoV-2 infection using a SARS-CoV-2 pseudovirus system and transcription and replication-competent SARS-CoV-2 virus-like-particles (trVLP). In addition, LF and MUC1 inhibited multiple steps including viral attachment, entry, and postentry replication, whereas α-LA inhibited viral attachment and entry. Importantly, LF, MUC1, and α-LA possess potent antiviral activities toward variants such as B.1.1.7 (alpha), B.1.351 (beta), P.1 (gamma), and B.1.617.1 (kappa). Taken together, our study provides evidence that human breast milk components (LF, MUC1, and α-LA) are promising antiviral and potential therapeutic candidates warranting further development for treating COVID-19.

INTRODUCTION

There is a greater threat to global health ever since the outbreak of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 shows a close phylogenetic relationship with SARS-CoV that caused an outbreak in 2002 (Meo et al., 2020). The high mortality rate of the pandemic has caused the health system to collapse, especially in developing countries (Guan et al., 2020). In addition, the ancestral virus has evolved into several viral variants through the course of the pandemic (Leung et al., 2021; Liu et al., 2021; Wu et al., 2021). These variants may have potentially altered viral transmission, pathogenicity, efficacy of drug treatment, as well as neutralizing capacity of vaccine-elicited antibodies (Gu et al., 2020; Ren et al., 2021; Starr et al., 2020; Volz et al., 2021). With this situation, it is urgent to develop effective drugs to treat this disease.

As reported previously, COVID-19 pandemic also confers a concern of the safety of breastfeeding at the beginning of its outbreak (Dong et al., 2020; Gross et al., 2020; Lugli et al., 2020; Unger et al., 2020; Yu et al., 2020). Despite detection of SARS-CoV-2 RNA in human breast milk, it is still unclear if SARS-CoV-2 could transmit from infected mothers to infants through breastfeeding (Gross et al., 2020). Several lines of evidence in the clinical studies showed that SARS-CoV-2 cannot transmit to infants via breast milk (Chambers et al., 2020; Lugli et al., 2020; Perrone et al., 2020; Unger et al., 2020; Yu et al., 2020). The World Health Organization and other organizers who drafted several guidelines still recommend continuing breastfeeding even if the mothers have the symptoms of COVID-19 (WHO, 2020; Wszolek et al., 2021). However, more information is needed to support breastfeeding.

Human milk is uniquely suited to feed infants because of its nutritional composition and bioactive factors promoting antimicrobial and immunomodulatory effects (van der Strate et al., 2001). Breast milk inhibits several viral infections, such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), and dengue virus. In addition to those enveloped viruses, breast milk also inhibits many non-enveloped viruses like rotavirus, enterovirus, and adenovirus (Kell et al., 2020; van der Strate et al., 2001). Lactoferrin (LF), an important component in breast milk, suppresses SARS-CoV and SARS-CoV-2 through blocking viral binding to

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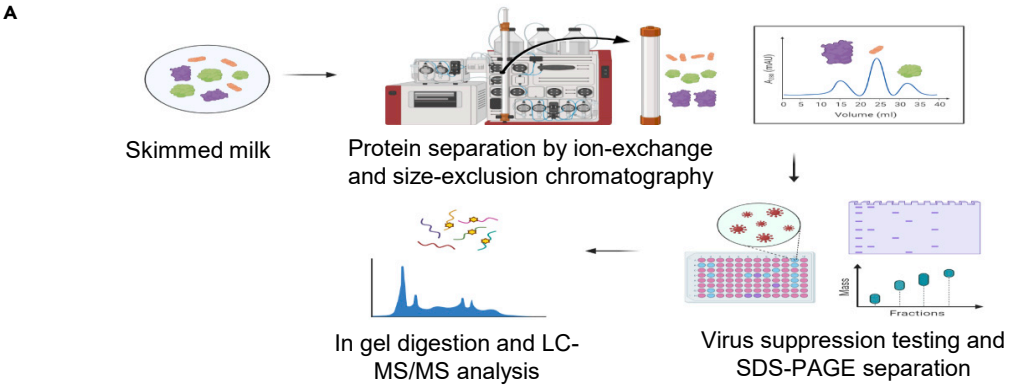

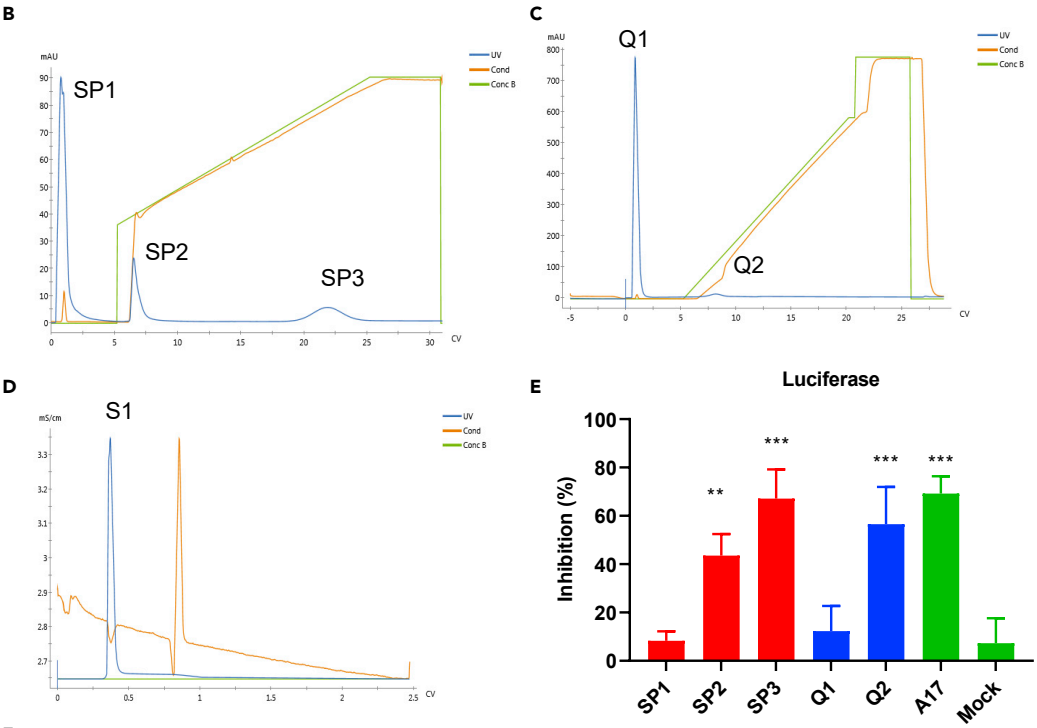

F

Cation-Exchange Chromatography Peak 3		Anion-Exchange Chromatography Peak 2			Size-Exclusion Chromatography	
Gene	Spectral Counts	Gene	Spectral Counts Exp1	Spectral Counts Exp2	Gene	Spectral Counts
LTF	110	LTF	88	186	LTF	79
ALB	24	ALB	3	10	ALB	8
IGHA1	11	IGHA1		7	IGHA1	4
H4C1	9	H4C1	3	7	H4C1	1
IGHA2	8	IGHA2	2	5	IGHA2	1
H2AC11	8	H2AC11	1	5	H2AC11	1

Figure 1. Identification of the key factors from skimmed milk for inhibiting SARS-CoV-2 infection by mass spectrometry

(A–D) Schematic illustration of identification of key factors from skimmed milk by mass spectrometry. The skimmed milk was separated through the cation exchange column (B), the anion exchange column (C), and the size exclusion column (D) in sequence. The UV signal indicates the protein fraction (280 nm).

(E) Inhibition analysis of the collected fractions to the inhibition of SARS-CoV-2 pseudovirus infection. Vero E6 cells were infected with 650 $TCID_{50}$/well of SARS-CoV-2 pseudovirus and treated with different fractions with the same ration at the same time. The human skimmed breast milk (A17) was used as a positive control. The cells were lysed and the luminescence was measured according to the manufacturer's instruction at 24 h postinfection.

(F) Table with top 6 (based on number of spectral counts) hits from skimmed milk in eluate fractions determined by tandem mass spectrometry (MS/MS). Data are presented as mean ± SD and repeated at least three times (n = 3), **p < 0.01, ***p < 0.001. See also Figures S1–S4.

heparan sulfate proteoglycans, raising the possibility that human breast milk can also suppress SARS-CoV-2 infection (Lang et al., 2011; Mirabelli et al., 2021; Hu et al., 2021). Indeed, we previously confirmed that human breast milk significantly inhibits SARS-CoV-2 and its related pangolin coronavirus (GX_P2V) infection. Importantly, we observed significantly higher anti-SARS-CoV-2 effects of breast milk than LF mono-treatment, indicating that other important factors in breast milk may contribute to the inhibition of SARS-CoV-2 infection and replication (Fan et al., 2020). Thus, it remains an urgent need to identify these factors in breast milk that have the potential to suppress SARS-CoV-2 infection.

Breast milk is a mixture of diverse nutrients that protect infants from different kinds of diseases. It contains more than 400 different proteins and many of them exhibit antimicrobial activity (Ballard and Morrow, 2013). Proteins in human milk can be divided into three groups, namely, caseins, mucin, and whey proteins. These bioactive proteins from the whey fraction include LF, mucins (MUC1 and MUC4), α-lactalbumin (α-LA), lactadherin, lactoperoxidase, IgA, lysozyme (LZ), and so on (Ballard and Morrow, 2013; Florisa et al., 2003). LF is an iron-binding protein with two molecules of Fe^{3+} ion per protein and rich in human milk (1–2 g/L in mature milk, 5–10 g/L in colostrum), whereas relatively lower level is present in other species such as bovine milk (0.02–0.3 g/L in mature milk, 2–5 g/L in colostrum) (Florisa et al., 2003). As reported previously, LF is an important component to protect infants from microbial infection (Florisa et al., 2003; van der Strate et al., 2001). LZ is also known to be a key protein inhibiting bacteria and rich in human milk (0.4 g/L). Lactadherin, a 46 KDa mucin-associated protein, was reported to display high viral receptor affinity and specific anti-rotavirus activity (Florisa et al., 2003). MUC1, mostly present in the fat globule membrane (cream fractions, about 1.32–1.41 g/mL) with very low level in skim milk (about 50 ug/mL), has the antiviral activity to inhibit influenza virus infection (McAuley et al., 2017; Mthembu et al., 2014). In addition, human milk also contains antibodies like immunoglobulin (Ig)G and IgA which show antiviral activity (Ejemel et al., 2020; Florisa et al., 2003). Therefore, these findings inspired us to identify the anti-SARS-CoV-2 components in breast milk and explore the underlying mechanisms.

Here, we aimed to identify the potential factors of human breast milk in suppressing SARS-CoV-2 infection by combing biochemical fractionation, activity assays, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Hu et al., 2014). Antiviral activities of individual fractions from breast milk were measured in multiple cell lines (Huh7.5, Vero E6, and Caco-2-N) infected by SARS-CoV-2 pseudovirus and trVLP system (Ju et al., 2021). Remarkably, we identified LF, MUC1, and α-LA with strong inhibitory effects on SARS-CoV-2 infection together with different variants by blocking viral entry and postentry replication. These findings shed light on the important roles of LF, MUC1, and α-LA derived from human milk and provide the basis for the rational design and development of antiviral drugs.

RESULTS

LF, MUC1, and α-LA were identified in skimmed milk with inhibition of SARS-CoV-2 infection

To identify the potential factors of human skimmed milk in suppressing SARS-CoV-2 infection, we designed the following experimental strategy (Figure 1A). Firstly, we performed biochemical fractionation by ion exchange chromatography. Then individual fractions were subjected to antiviral activity assays (Figures 1B–1D), where Vero E6 cells were infected with 650 $TCID_{50}$/well of SARS-CoV-2 pseudovirus with the addition of fractionated samples in the media. One day postinfection, we measured their inhibitory effects on SARS-CoV-2 infection (Figure 1E). The fraction that retained the highest activity was analyzed by LC-MS to identify the potential factors from the skimmed milk shown in Figure 1A. Briefly, we first processed skimmed milk for ammonium sulfate precipitation to remove most proteins without antiviral activity. After precipitation with different concentrations of ammonium sulfate, we assayed the supernatants for their inhibition of viral infection. We found that most of the ingredients with antiviral activity were retained in the supernatant with the use of 1 M ammonium sulfate. After precipitation, the samples were fractionated by sequential

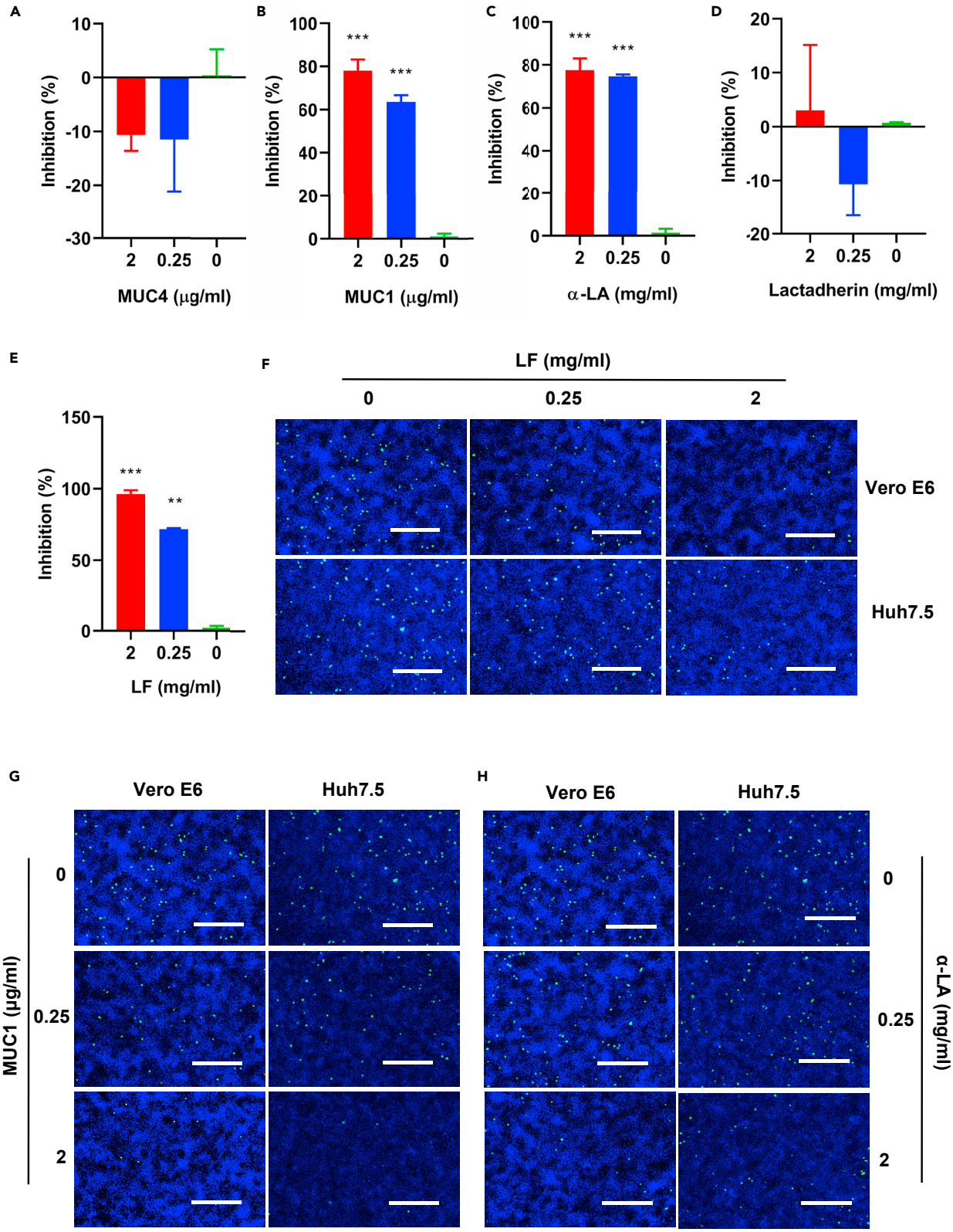
A
Inhibition (%)
MUC4 (μg/ml)
B
Inhibition (%)
MUC1 (μg/ml)
C
Inhibition (%)
α-LA (mg/ml)
D
Inhibition (%)
Lactadherin (mg/ml)
E
Inhibition (%)
LF (mg/ml)
F
LF (mg/ml)
Vero E6
Huh7.5
G
Vero E6
Huh7.5
MUC1 (μg/ml)
H
Vero E6
Huh7.5
α-LA (mg/ml)

Figure 2. Identification of the key factors from the reported skimmed milk's factors for inhibiting SARS-CoV-2 infection

Inhibition analysis of MUC4 (A), MUC1 (B), α-LA (C), lactadherin (D), and LF (E) to SARS-CoV-2 pseudovirus infection. Vero E6 cells were infected with 650 $TCID_{50}$/cells of SARS-CoV-2 pseudovirus and treated with different concentrations of MUC4, MUC1, α-LA, lactadherin, and LF at the same time. The cells were lysed and the luminescence was detected according to the manufacturer's instruction at 24 h postinfection. Fluorescence analysis of different concentrations of LF (F), MUC1 (G), and α-LA (H) to inhibit SARS-CoV-2 pseudovirus with GFP expression in both Vero E6 and Huh 7.5 cells. Scale bar represents 100 μm α-LA, a-lactalbumin; LF, and lactoferrin. Data are presented as mean ± SD and repeated at least three times (n = 3), $^{**}p < 0.01$, $^{***}p < 0.001$.

ion exchange chromatography. The active fractions from each column step were pooled, concentrated, dialyzed, and loaded onto the next column. We fractionated the separated supernatant by using cation exchange chromatography and collected both the flow-through (SP1) and column-bound fractions eluted with a linear salt gradient from 0 M to 1 M of NaCl (SP2 and SP3) (Figure 1B). We observed highest antiviral activity at fraction SP3 (Figure 1E), which was concentrated, dialyzed, and subjected further to anion exchange chromatography. After collection of both the flow-through (Q1) and column-bound fractions eluted with 1M NaCl (Q2) (Figure 1C), we found the antiviral activity was retained at fraction Q2 but not Q1. Next, we ran the Q2 fraction by SDS-PAGE (Figure 1D), and the stained protein bands between 70 and 100 kDa were excised and processed for mass spectrometric identifications. Eventually, we found that LF in skimmed milk was a potential factor for SARS-CoV-2 inhibition (Figures 1F and S1–S4).

As reported previously, LF might not be the effective factor alone in breast milk and there might be other components showing anti-SARS-CoV-2 activity. Previous reports showed that some factors, such as MUC1, MUC4, lactadherin, α-LA, etc., have antimicrobial activity (Ballard and Morrow, 2013; Florisa et al., 2003). To identify the factors that also inhibit SARS-CoV-2 infection, we mixed the MUC4, MUC1, α-LA, lactadherin, and LF with 650 $TCID_{50}$/well of luciferase-expressing SARS-CoV-2 pseudovirus, respectively. Then, the mixture was used to infect the Vero E6 cell for 24h. MUC4 (Figure 2A) and lactadherin (Figure 2D) showed no suppression of SARS-CoV-2 pseudovirus infection. Interestingly, MUC1 (0.25 μg/mL, Figure 2B), α-LA (0.25 mg/mL, Figure 2C) and LF (0.25 mg/mL, Figure 2E) showed a dramatic inhibition of SARS-CoV-2 pseudovirus infection. In addition, we also used the GFP-expressing SARS-CoV-2 pseudovirus to verify the inhibition activity of LF, MUC1, and α-LA in both Huh7.5 and Vero E6 cell lines. As shown in Figures 2F–2H, all of them suppressed GFP expression, indicating that these factors in skimmed milk could inhibit SARS-CoV-2 infection. Thus, we identified that these three components in breast milk inhibit SARS-CoV-2 pseudovirus infection.

LF, MUC1, and α-LA suppress SARS-CoV-2 infection and replication in the trVLP-infected Caco-2-N cell system

To further explore to what extent these factors inhibit SARS-CoV-2 infection, we evaluated the effects of LF, MUC1, and α-LA on SARS-CoV-2 infection in a cell culture system of transcription-competent and replication-competent SARS-CoV-2 virus-like-particles (trVLP), which expresses a reporter gene (GFP) replacing viral nucleocapsid gene (N) and also could complete viral life cycle in the cells expressing N protein (Ju et al., 2021). Caco-2-N cells were infected with trVLP (MOI = 1) and treated with LF, MUC1, and α-LA at different concentrations for 96 h (Figure 3A). Subsequently, we carried out RT-qPCR analysis of cell lysates, in which a dramatic decrease in the levels of SARS-CoV-2 RNA upon treatment was observed (Figure 3B). Furthermore, GFP expression as a marker of viral replication revealed that both LF and MUC1 significantly suppressed SARS-CoV-2 replication (Figure 3C). We observed a relatively lower inhibitory effect of α-LA on SARS-CoV-2 infection, because some GFP expression was even seen at the concentration of 2 mg/mL. Consistent with the GFP results, western blot analysis of the cell lysates showed that MUC1 and LF could significantly suppress the S protein expression of SARS-CoV-2 (Figure 3D). The α-LA at a high concentration of 2 mg/mL could also suppress SARS-CoV-2 infection.

In addition, we examined whether LF orthologs from other species possess inhibitory effects on SARS-CoV-2 infection. We included the inhibitory effect of recombinant human LF (rLF), human-isolated LF (hLF), bovine LF (bLF), and goat LF (gLF) on SARS-CoV-2 infection assays with skimmed milk (fraction A17) as a positive control. Firstly, we analyzed the amino acid sequence of LF from human, bovine, and goat and found that their sequences are quite different, indicating a potential nonidentical antiviral activity (Figure 4A). Then, we utilized 650 $TCID_{50}$/well of SARS-CoV-2 pseudovirus to infect Vero E6 cells treated with rLF, hLF, bLF, gLF, and A17 for 24h. Afterward, we measured the luciferase reporter activity and found that all the LFs and A17 significantly suppressed SARS-CoV-2 pseudovirus infection regardless of their origin (Figure 4B). Consistent with the pseudovirus system, the data from the trVLP system supported

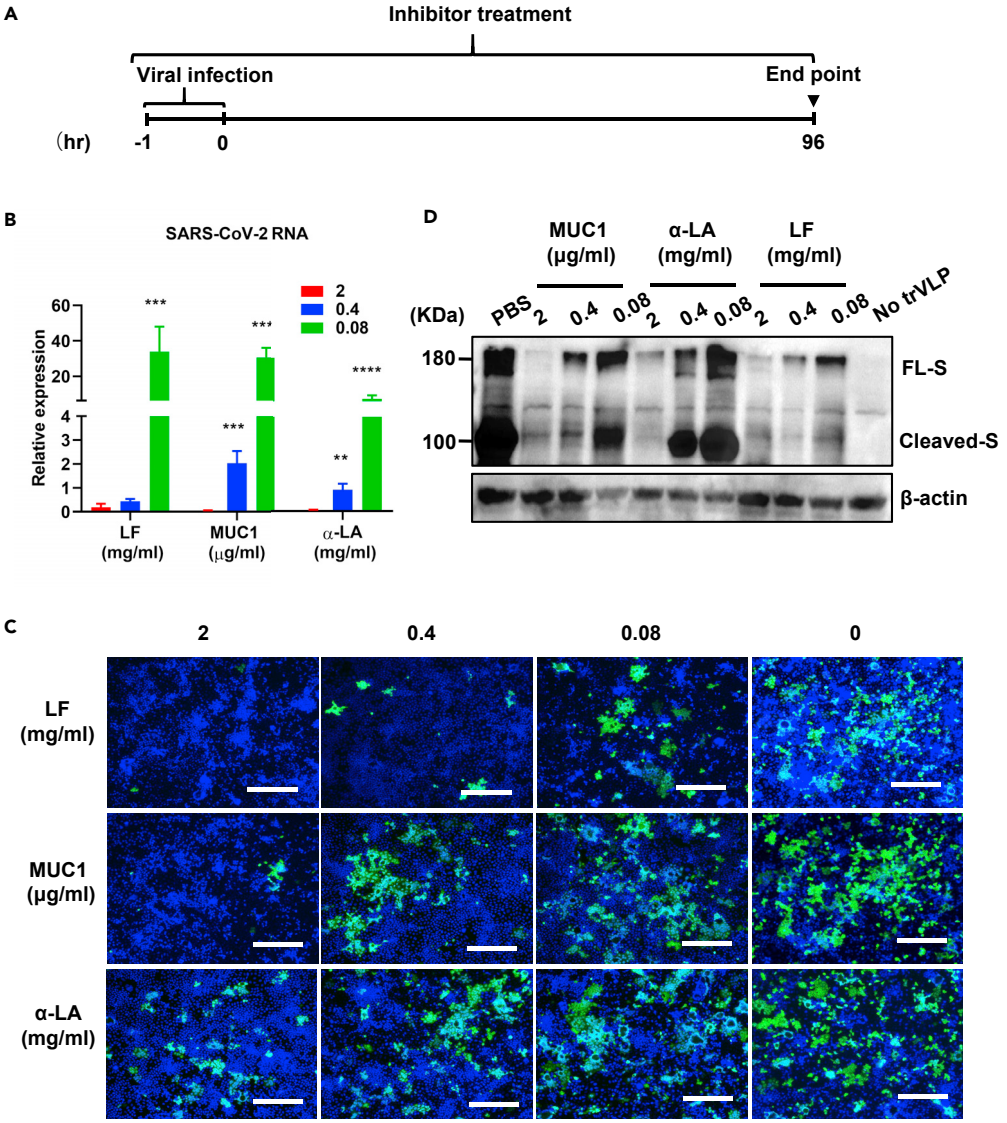

Figure 3. MUC1, a-lactalbumin, and lactoferrin suppress viral infection and replication in SARS-CoV-2-infected Caco-2-N cells

(A) Schematic illustration of the experiment.

(B) RT-qPCR analysis, (C) fluorescence analysis, and (D) western blot analysis were used to detect viral RNA, GFP, and viral spike protein in SARS-CoV-2-infected Caco-2-N cells treated with different doses of MUC1, α-LA, and LTF. Caco-2-N cells were infected with trVLP (SARS-CoV-2 N protein deficient particles, MOI = 1) and treated with different doses of LF, MUC1, and α-LA. α-LA, a-lactalbumin; LF, lactoferrin. Scale bar represents 100 μm. Data are presented as mean ± SD and repeated at least three times (n = 3), **p < 0.01, ***p < 0.001, ****p < 0.0001.

the inhibition of SARS-CoV-2 infection and replication by all the LFs as well when both the mRNA levels (Figure 4C) and GFP expression (Figure 4D) were downregulated. Western blot analysis of the cell lysates showed that rLF and bLF significantly suppressed SARS-CoV-2 S protein expression (Figure 4E). In addition, we found that gLF at high concentrations (i.e., 2 mg/mL) was toxic to cells (Figure 4F), whereas other LFs showed undetectable toxicity. While treating the cells at different concentrations and infecting them with trVLP (MOI = 1) for 96 h, we found that EC_{50} value of gLF for suppressing SARS-CoV-2 infection is low. However, CCK8 analysis showed that gLF at higher concentrations exhibited more robust cytotoxicity to Caco-2-N cells with CC_{50} of 5.64 mg/mL (Figure 4G). Together, we have shown that LF, MUC1, and α-LA from human breast milk are able to suppress SARS-CoV-2 infection and replication. In addition, LF from other species can also inhibit SARS-CoV-2 infection.

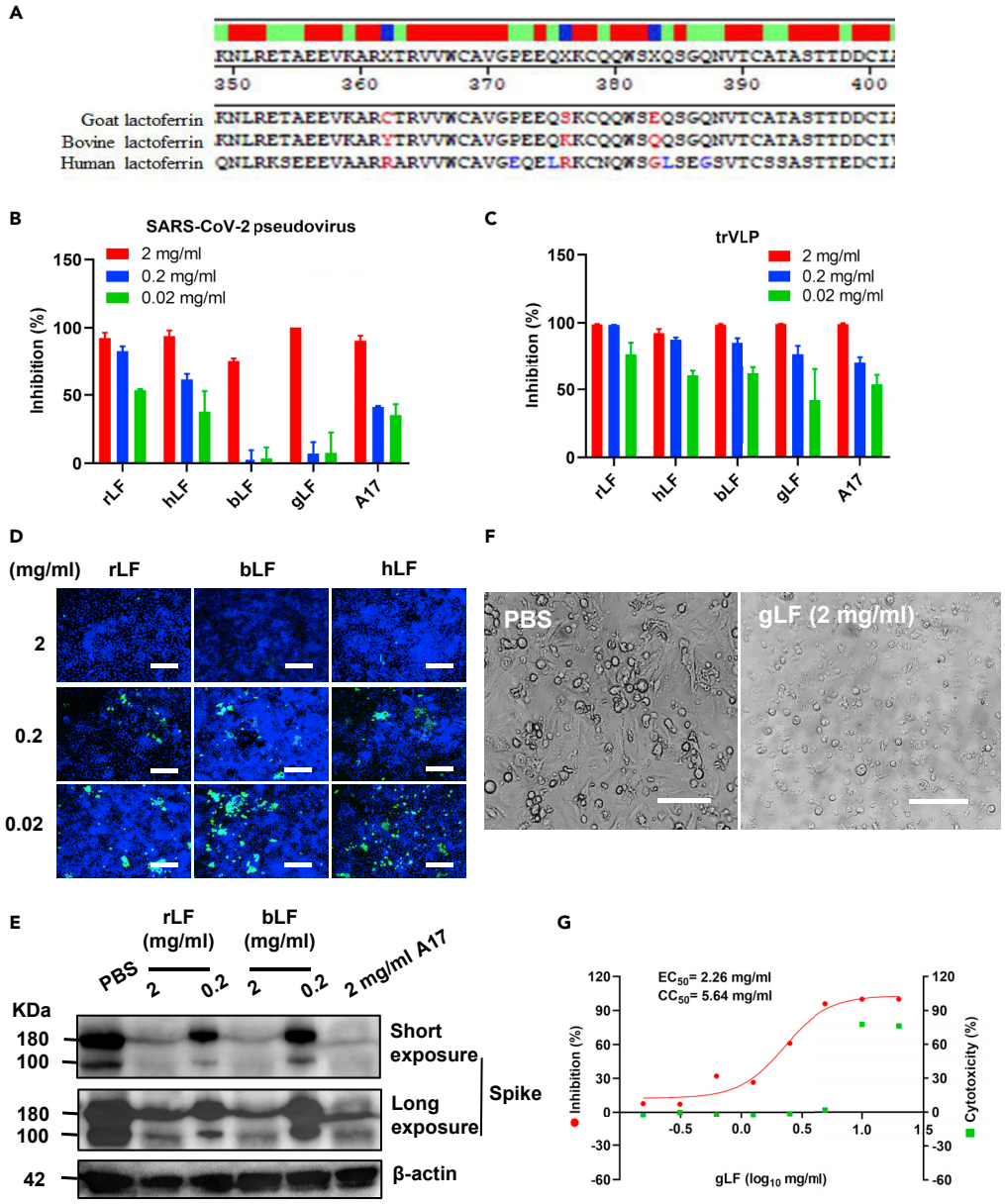

Figure 4. LF from different species also suppressed SARS-CoV-2 infection and replication

(A) Alignment analysis of LF from human, bovine, and goat.

(B–E) Inhibition analysis of recombinant LF (rLTF), human LF (hLF), bovine LF (bLF), and goat LF (gLF) to SARS-CoV-2 pseudovirus infection in Vero E6 cells. Skimmed milk (A17) was used as positive control for SARS-CoV-2 inhibition. RT-qPCR (C), immunoplot, (D) and western blot (E) were used to detect viral RNA, GFP, and viral spike protein in trVLP-infected Caco-2-N cells treated with different doses of rLF, hLF, bLF, gLF, and A17.

(F) Toxicity of high concentration of gLF in Vero E6 cell model.

(G) The gLF treatment dose dependently suppressed viral infection and replication, but with cell toxicity at high doses, in trVLP-infected Caco-2-N cells. Scale bar represents 100 μm. Data are presented as mean ± SD and repeated at least three times (n = 3), **p < 0.01, ***p < 0.001, ****p < 0.0001.

LF, MUC1, and α-LA dose and time dependently decrease SARS-CoV-2 infection and replication in association with reduced viral RNA

We performed concentration-response experiments with LF, MUC1, and α-LA to assess their suppression on SARS-CoV-2 infection and replication. Infection of Caco-2-N cells by the trVLP confirmed the

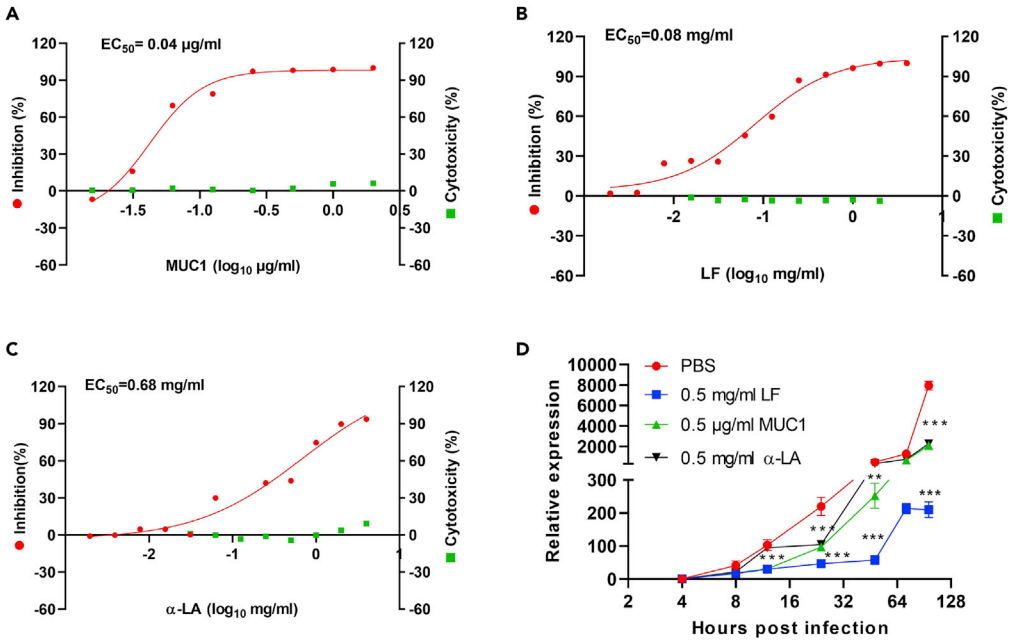

Figure 5. MUC1, LF, and α-LA treatment dose dependently suppressed viral infection and replication with reduced viral RNA in trVLP-infected Caco-2-N cells

The inhibition analysis reflected by intracellular viral RNA of trVLP-infected Caco-2-N cells treated with serial doses of MUC1 (A), LF (B), and α-LA (C) were determined by RT-qPCR. (D) The viral RNA levels with time dependence suppressed by MUC1, LF, and α-LA were detected by RT-qPCR. The Caco-2-N cells were infected with trVLP (MOI = 1) and treated with MUC1, LF, and α-LA at the same time, then the cells were harvested at different time points as designed. The cells were lysed and detected by RT-qPCR. Data are presented as mean ± SD and repeated at least three times (n = 3), ***$p < 0.001$. See also Figure S5.

concentration-dependent inhibitory effects of MUC1, LF, and α-LA on SARS-CoV-2 infection, and the 50% effective concentration (EC_{50}) for MUC1, LF, and α-LA was as low as 0.04 μg/mL (Figure 5A), 0.08 mg/mL (Figure 5B), and 0.68 mg/mL (Figure 5C), respectively. Surprisingly, the cytotoxic values evaluated by CCK-8 assay revealed no cytotoxicity of MUC1, LF, and α-LA to the cells, even at the highest concentrations of MUC1 (2 μg/mL), LF (2 mg/mL), and α-LA (2 mg/mL). Consistent with these results, the data from Vero E6 cells infected by SARS-CoV-2 pseudovirus shown in Figure S5A further confirmed the inhibitory effects of MUC1 on SARS-CoV-2 pseudovirus infection with the EC_{50} for MUC1 as low as 0.1 μg/mL. Similarly, we observed fairly low EC_{50} values of LF and α-LA as well (0.1 mg/mL (Figure S5B) and 0.16 mg/mL (Figure S5C), respectively).

While treating the cells with low concentration of MUC1, LF, and α-LA in the trVLP-infected Caco-2-N cells, we found that all three factors still significantly suppressed SARS-CoV-2 infection at an early time point. Besides, such inhibitory effects were sustained for both LF and MUC1 during the culture period (Figure 5D). Although, α-LA inhibited SARS-CoV-2 infection at early time points, the extent of inhibition declined over time, suggesting that α-LA only interferes with the early steps of the viral life cycle, such as viral attachment and entry.

LF and MUC1 adversely affect viral attachment, entry, and postentry of viral replication, yet α-LA only decreases viral attachment and entry

To explore which stage of SARS-CoV-2 replication cycle is targeted by LF, MUC1, and α-LA, we treated the Caco-2-N cells with individual proteins at different time points pre-viral and post-viral infection. The attachment experiments were carried out as shown in Figure 6A (preinfection treatment), in which these factors were washed away before viral infection. We observed a significant decrease of viral RNA in those groups treated by MUC1, α-LA, rLF, hLF, bLF, and A17 (Figure 6B). Similarly, cells treated with these factors showed nearly no viral replication associated with GFP expression at all (Figure 6C). We also performed an infection using pseudovirus (Figure S6A) and found comparable reduction of the luciferase expression in Vero E6

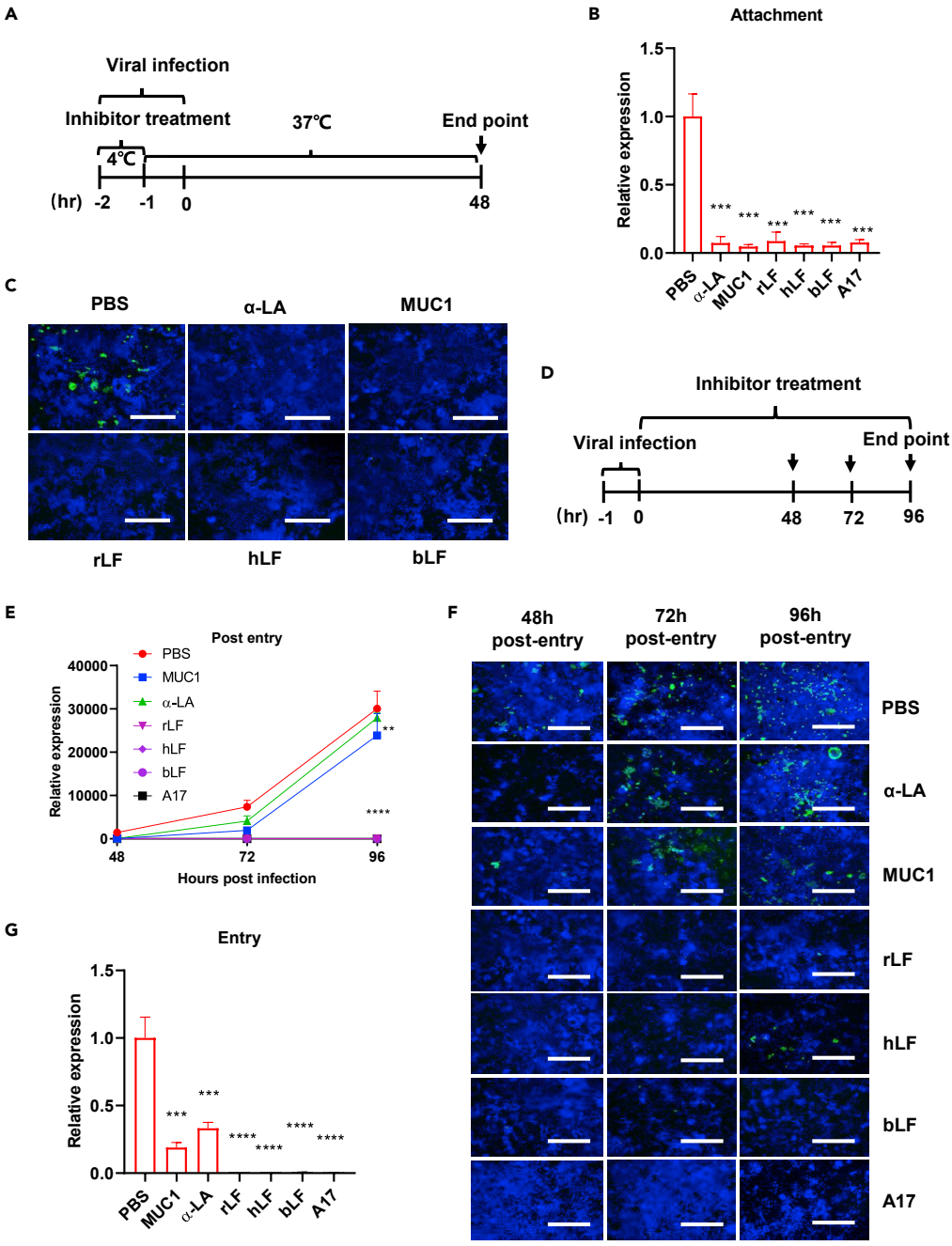

Figure 6. MUC1, LF, and α-LA treatment affects the early stage of viral infection including attachment and entry, but LF also decreases viral RNA levels after virus entry

(A–C) Schematic illustration of the pretreatment experiment. RT-qPCR (B) and immunoblot (C) assays were used to detect viral RNA and GFP expression in trVLP-infected Caco-2-N cells. Skimmed milk (A17) was used as positive control for inhibiting SARS-CoV-2 infection.

(D–F) schematic illustration of the postinfection treatment experiments. RT-qPCR (E) and immunoblot (F) assays were used to detect viral RNA and GFP expression at different time points in trVLP-infected Caco-2-N cells.

(G) The viral RNA levels detected by RT-qPCR are suppressed by MUC1, α-LA, rLF, hLF, bLF, and A17 during viral entry. The trVLP was mixed with Caco-2-N cells at 4°C for 1 h then discarded the supernatant and washed with PBS for three times. The cells were then added with fresh media with MUC1, α-LA, rLF, hLF, bLF, and A17 treatment at 37°C for 2 h. After that, the supernatant was discarded and the cells were reloaded with fresh media and cultured for 48 h. The cells were lysed and the viral RNA was detected by RT-qPCR. MUC1 (2 ug/mL), α-LA (a-lactalbumin, 2 mg/mL), rLF (recombinant

Figure 6. *Continued*
lactoferrin, 2 mg/mL), hLF (human lactoferrin, 2 mg/mL), bLF (bovine lactoferrin, 2 mg/mL), and A17 (skimmed milk, 2 mg/mL). Scale bar represents 100 μm. Data are presented as mean ± SD and repeated at least three times (n = 3), ***p < 0.001, ****p < 0.0001. See also Figure S6.

and Huh7.5 cells (Figure S6B). Taken together, these data indicated that MUC1, LF, and α-LA might block viral attachment and entry.

Next, we tested their effects on the postinfection experiment as shown in Figure 6D. In these experiments, we found a reduced level of the viral RNA and GFP expression upon treatment with MUC1 and LF, whereas cells treated with α-LA showed comparable levels of RNA and GFP expression to controls (Figures 6E and 6F). In line with these data, we observed significantly lower luciferase expression of pseudovirus (Figure S6C) after MUC1 and LF treatment, whereas cells treated with α-LA only exhibited a minor reduction (Figure S6D). Given that the pseudovirus was packaged based on the VSV, the postentry replication usually reflects VSV replication. Then, we included the VSV as a control in the designed experiment (Figure S6C). As expected, these factors also inhibited VSV replication (Figure S6E). These data suggested that both LF and MUC1, but not α-LA, can suppress SARS-CoV-2 postentry replication.

In addition, we also examined whether MUC1, LF, and α-LA can suppress viral entry. We infected the Caco-2-N cells with trVLP and Huh7.5 and Vero E6 cells with pseudovirus at 4°C for 1 h, respectively. Then, we washed the virus away and refreshed the media supplemented with these factors until the endpoint of infection. Interestingly, all these factors showed potent antiviral activity as evidenced by the dramatic reduction of the SARS-CoV-2 RNA (Figure 6G) and luciferase activity levels (Figure S6F). Together, these data suggested that MUC1, LF, and α-LA suppress SARS-CoV-2 entry step of viral life cycle.

The α-LA interferes with the interaction of ACE-2 and S protein, whereas MUC1 and LF suppress SARS-CoV-2 attaching to the HSPG

Next, we sought to determine if MUC1, LF, and α-LA can interfere with the interaction of ACE-2 and S protein by performing the affinity assays. As shown in Figures 7A and 7B, LF and MUC1 did not block the interaction of ACE-2 and S protein, indicating that they suppress SARS-CoV-2 infection by mechanisms independent of the ACE-2 pathway. Interestingly, our assays revealed that α-LA interfered with the association between SARS-CoV-2 spike protein and ACE-2, as evidenced by high absorbance after α-LA treatment at a low concentration of 0.25 mg/mL (Figure 7C).

Moreover, it was reported that LF inhibits SARS-CoV infection by targeting HSPG (Lang et al., 2011). To determine its potential involvement in the inhibition of SARS-CoV-2 infection, we treated the cells with LF combined with different concentrations of heparin. We found that the inhibitory effect of LF and MUC1 on SARS-CoV-2 decreased with the addition of heparin in a concentration-dependent manner, to the deep when 10 U/mL of heparin was added in both Huh7.5 (Figures 7D and 7F) and Vero E6 (Figures 7E and 7G) cells. However, when the level of heparin was increased to 100 U/mL, we found a more potent inhibitory effect, indicating that heparin could interfere with the inhibitory effect of LF on SARS-CoV-2 infection.

To further confirm that LF and MUC1 interact with SARS-CoV-2 attaching to HSPG, we also performed new experiments in the trVLP-infected Caco-2-N cells as designed in Figure S7A. As shown, we also found that MUC1 (Figure S7B) and LF (Figure S7C) rather than α-LA (Figure S7D) could interfere with heparin's inhibition of SARS-CoV-2 infection. These data suggested that inhibition of viral attachment to HSPG contributes, at least partially, to LF-suppressing SARS-CoV-2 infection.

MUC1, LF, and α-LA suppress the infection and replication of several common SARS-CoV-2 mutants

To determine whether these factors are effective in inhibiting SARS-CoV-2 variants, we constructed and packaged four SARS-CoV-2 mutants (i.e., B.1.1.7 (alpha), B.1.351 (beta), P.1 (gamma), and B.1.617.1 (kappa)) based on the trVLP system. Caco-2-N cells were infected with these variants and treated with different concentrations of MUC1, rLF, and α-LA, respectively (Figure 8A). After 48 h of infection, the cells were harvested. Interestingly, MUC1, rLF, and α-LA retained their ability to suppress SARS-CoV-2 variants in a dose-dependent manner (B.1.1.7 (alpha) (Figure 8B), B.1.351 (beta) (Figure 8C), P.1 (gamma)

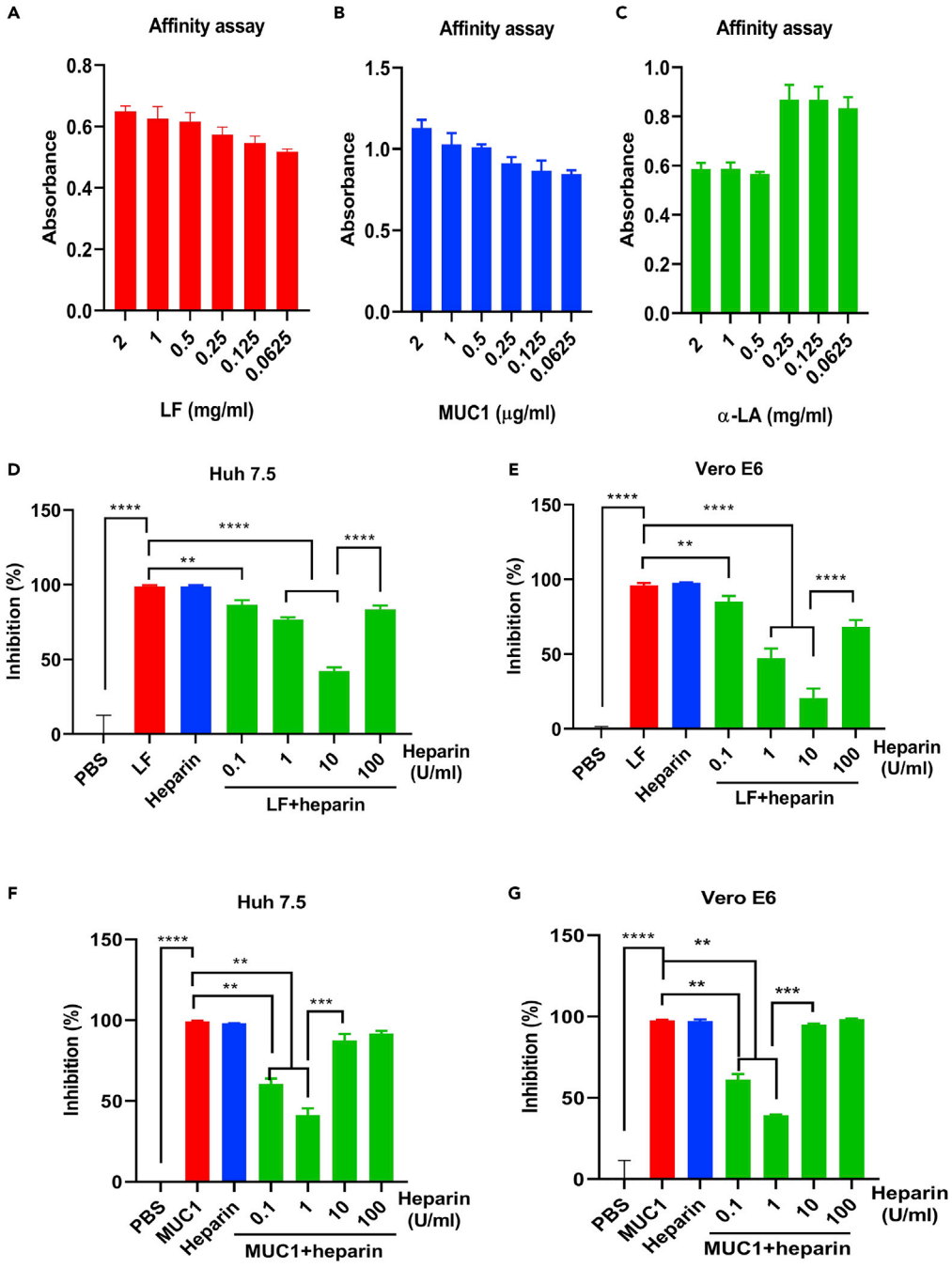

Figure 7. α-LA interferes the affinity of spike protein and ACE-2, MUC1, and LF inhibits SARS-CoV-2 attaching to HSPG

Affinity assay for the impact of LF (A), MUC1 (B), and α-LA (C) on the affinity between ACE2 and SARS-CoV-2 RBD. (D) and (E) inhibition of SARS-CoV-2 pseudovirus infection. Huh7.5 and Vero E6 cells were treated with heparin for 10 min at the dosage of 0.1 U/mL, 1 U/mL, 10 U/mL, and 100 U/mL. Then, 2 mg/mL LF was added to each group and incubated at 37°C for 1h. Similarly, (F) and (G) inhibition of SARS-CoV-2 pseudovirus infection. Huh7.5 and Vero E6 cells were treated with heparin for 10 min at the dosage of 0.1 U/mL, 1 U/mL, 10 U/mL, and 100 U/mL. Then, 2 mg/mL LF was added to each group and incubated at 37°C for 1h. The luciferase assay was performed to detect SARS-CoV-2 pseudovirus infection. Data are presented as mean ± SD and repeated at least three times (n = 3), **p < 0.01, ****p < 0.0001. HSPG: heparan sulfate proteoglycans; LF: lactoferrin; MUC1: mucin 1. See also Figure S7.

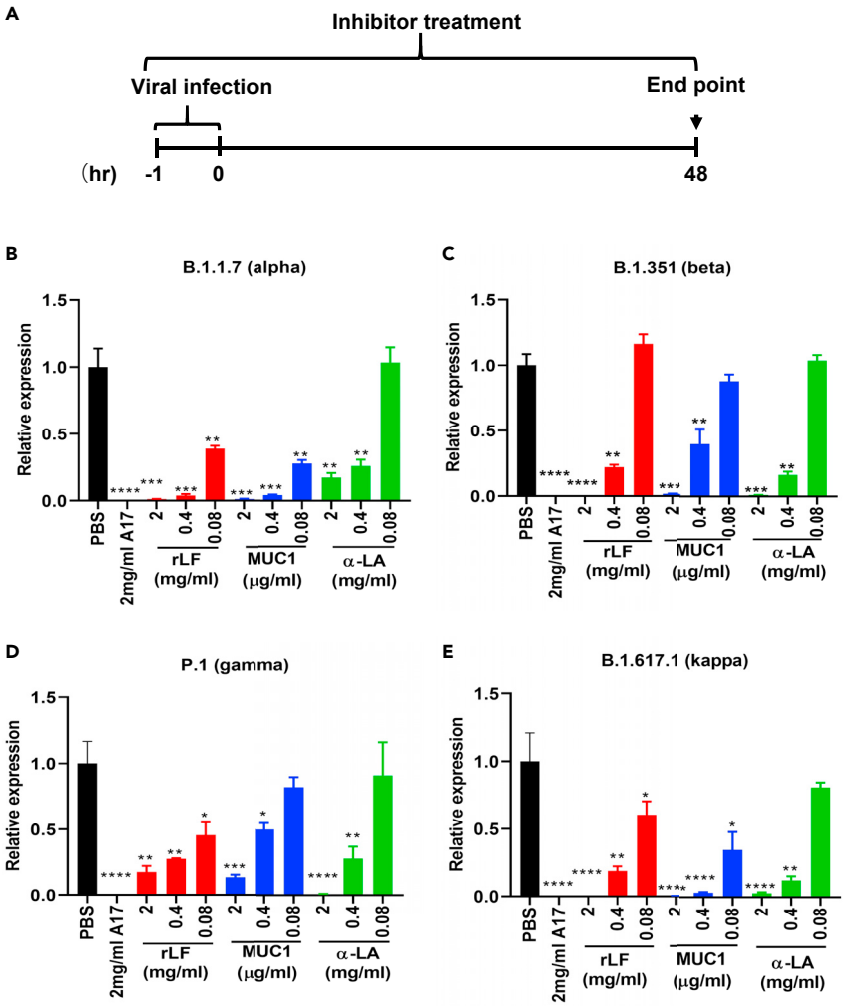

Figure 8. MUC1, LF, and α-LA suppress different SARS-CoV-2 variants infection and replication in Caco-2-N cells

(A) Schematic illustration of the treatment experiment of MUC1, LF, and α-LA for SARS-CoV-2 variants infection. Caco-2-N cells were seeded in six well plates and infected with SARS-CoV-2 variants of (B)1.1.7 (alpha), (B)1.351 (beta), P.1 (gamma), and (B)1.617.1 (kappa) (MOI = 0.1). The cells were treated with MUC1, rLF, and α-LA, respectively during the infection. PBS and skimmed milk fraction A17 were used as negative and positive control, respectively. Forty-eight hours postinfection, the cells were harvested and tested. The inhibition analysis of MUC1, rLF, and α-LA to SARS-CoV-2 variants of (B) (B)1.1.7 (alpha), (C) (B)1.351 (beta), (D) P.1 (gamma), and (E) (B)1.617.1 (kappa) reflected by intracellular viral RNA were determined by RT-qPCR. Data are presented as mean ± SD and repeated at least three times (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. α-LA, a-lactalbumin; LF, lactoferrin; A17, skimmed milk.

(Figure 8D), and B.1.617.1 (kappa) (Figure 8E)). These results suggested that MUC1, rLF, and α-LA are able to suppress the infection and replication of SARS-CoV-2 variants as well.

DISCUSSION

In this study, we developed an experimental workflow combining biochemical fractionation, LC-MS, and SARS-CoV-2 pseudovirus system profiling that allows effective screening of the potential components in human breast milk for inhibiting SARS-CoV-2 infection *in vitro*. Of these, we identified and confirmed that LF, MUC1, and α-LA in the breast milk significantly inhibited SARS-CoV-2 pseudovirus infection in Huh7.5 and Vero E6 cells and trVLP infection in Caco-2-N cells. It was confirmed that LF and MUC1 play a critical role not only in blocking viral attachment and entry into host cells but also in suppressing postentry replication at multiple cell lines. In comparison, α-LA could only block viral attachment and entry into the cells. These data shed light on the groundwork for further developing antiviral treatment to SARS-CoV-2 infection and spread.

Despite the lack of solid clinical evidence of COVID-19-related MTCT through breastfeeding, SARS-CoV-2 positive in breast milk still confers a great concern of the safety of breastfeeding to infants from SARS-CoV-2 infected mothers (Chambers et al., 2020; Chen et al., 2020; Dong et al., 2020; Zhu et al., 2020). Chambers et al. reported that they collected the positive breast milk from 18 infected women and tested the infectivity in the cell culture system (Chambers et al., 2020). They did not find any viral RNA positive in the experiments, indicating that SARS-CoV-2 in the breast milk cannot infect and transmit. Consistent with this, previously, we have also shown that even if we added the live virus at relatively high concentrations in the breast milk, the virus still could not infect the cells, suggesting highly potent activity of human breast milk in anti-SARS-CoV-2 infection (Fan et al., 2020). Therefore, the international guidelines and World Health Organization still strongly recommend to continue breastfeeding even if mothers have symptoms of COVID-19, because breastfeeding unquestionably reduces neonatal and infant mortality and provides numerous lifelong health and brain development advantages to the infants (WHO, 2020; Wszolek et al., 2021). In this study, we successfully identified LF, MUC1, and α-LA in the milk with highly effective antiviral activity to SARS-CoV-2 infection. Interestingly, the EC_{50} values of these factors are much lower than their concentrations in human breast milk, suggesting the strong antiviral activity of human breast milk for SARS-CoV-2 infection. As a result, hardly any live SARS-CoV-2 virus in the milk can infect and transmit to infants during breastfeeding. For example, the EC_{50} value of LF to SARS-CoV-2 is 0.08 mg/mL, whereas the concentration of LF in human mature breast milk and colostrum is 1–2 mg/mL and 5–10 mg/mL, respectively. In other words, such high LF concentrations in breast milk effectively inhibit potential SARS-CoV-2 infection and transmission. This analysis suggested that breastfeeding may be safe for infants whose mothers are infected with SARS-CoV-2, if the breast milk is pumped into sterile containers and infants are fed in isolation from their mothers. Thus, our data provides strong evidence on the importance of breast milk in antiviral property, which strongly supports the guidelines for managements of birth and breastfeeding during SARS-CoV-2 pandemic. However, further studies are still warranted to confirm the safety of breastfeeding to infants from SARS-CoV-2 infected mothers.

SARS-CoV-2 is able to mutate simply in its RNA genome during the infection, leading to viral escape from antibody neutralization, vaccination, and other drugs targeting the interaction between S protein and ACE-2 (Liu et al., 2021). Surprisingly, we provided evidence that LF, MUC1, and α-LA are still highly effective in inhibiting the currently transmitting mutants such as B.1.1.7 (alpha), B.1.351 (beta), P.1 (gamma), and B.1.617.1 (kappa). Therefore, we conclude that these factors from breast milk can offer strong and broad-spectrum protection from SARS-CoV-2 infection.

It is noteworthy that LF, MUC1, and α-LA identified in our study are also safe and available in dietary supplements (Andreas et al., 2015). All these factors were reported with antiviral activity and their expression was associated with viral infection (Florisa et al., 2003). Reghunathan R et al. showed upregulated expression of LF expression during SARS-CoV infection blocked viral attachment through HSPG (Reghunathan et al., 2005). Moreover, Mirabelli C et al. reported that LF screened from the FDA approved drugs also inhibited SARS-CoV-2 to bind to the cells through competition with HSPG and also modulate innate immune responses to increase expression of interferon-stimulated genes and TNFα (Mirabelli et al., 2021). Similarly, our study also proved that LF from different species (such as bovine and goat) also inhibited SARS-CoV-2 attachment to HSPG and postentry replication. LFs from human and bovine showed nearly no toxicity to all used cells such as, Huh7.5, Vero E6, and Caco-2-N cells. However, gLF could be toxic to cells at high concentration treatment, indicating variations from different species. Our previous study showed that skimmed milk can suppress the activity of RNA-dependent RNA polymerase of SARS-CoV-2 (Fan et al., 2020). Thus, further studies are required to elucidate the exact mechanism(s) of how LF inhibits SARS-CoV-2 infection and replication.

In addition, MUC1 is an important factor expressing in the lung airway, maintaining lung function and health (Dhar and McAuley, 2019). MUC1 plays an important role in protection of the host from infection by pathogens and regulating inflammatory response as well (Dhar and McAuley, 2019). Lu W et al. reported increased MUC1 expression in the airway mucus of severely sick COVID-19 patients (Lu et al., 2021). In addition, MUC1 sialylated on the cell surface has the potential ability to bind influenza A virus (IAV), thereby reducing the viral infection of host cells (McAuley et al., 2017). Consistent with these findings, our data showed that MUC1 from milk could inhibit SARS-CoV-2 infection through influence on viral attachments, entry and postentry replication. However, an affinity assay showed MUC1 did not interfere with viral infection based on the ACE-2 target, indicating the other potential mechanisms are involved in the infection. Interestingly, we found that the recombinant MUC1 C-terminal truncated (MUC1-C) without any variable number tandem repeat

(VNTR) region also showed high inhibitory activity to SARS-CoV-2 infection, reinforcing the notion of the mechanism for MUC1 to inhibit SARS-CoV-2 infection and replication. Many studies demonstrated that MUC1 plays an important role in inhibiting Toll-like receptor and NOD-like receptor protein 3-dependent inflammation (Dhar and McAuley, 2019). These results suggested that MUC1 could be used as a potential drug candidate for inhibiting SARS-CoV-2 infection and related inflammation to reduce the severity of COVID-19. However, further studies are needed. As for α-LA, it seems that this protein can only block viral attachment to the cells through interfering with viral binding to the ACE-2 receptor.

Collectively, we found that several factors from human breast milk (LF, MUC1, and α-LA) effectively inhibit the infection and replication of SARS-CoV-2 and their variants by blocking viral attachment, entry, and post-entry replication. These promising findings showed the safety of breastfeeding in the clinic; nonetheless, further studies are still needed to confirm our data in both animal and clinical studies and further modification of these proteins is necessary for potential clinical usage.

Limitations of the study

Although lactoferrin, mucin1, and α-lactalbumin identified from breast milk in this study hold a promising potential to be used as a clue for the safety of breastfeeding when the mother is infected with SARS-CoV-2 and also to be developed as inhibitors of SARS-CoV-2 infection, there are a few limitations that need to be addressed before their further application. Firstly, this study was based on the *in vitro* cell models, which needed to be tested *in vivo* efficacy and toxicity. In addition, it would be helpful to test the efficacy of these factors in inhibiting viral infection of different variants from clinics. Mechanistically, this study suggests that lactoferrin, mucin1, and α-lactalbumin interfere with viral attachment, entry, and postentry replication. It would be important to identify host factors and innate immune responses that these factors interact with to inhibit SARS-CoV-2 infection in the future.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

K.X. designed the research. X.Lai., Y.Y., W.X., F.Y., X.J., Y.L., T.L., and H.D. performed the experiments. K.X., X.Lai, Y.Y., W.X., Q.D., and X.Liu analyzed the data. K.X., Q.D., T.L., H.Z., Y.Z., W.T., and X.Liu wrote and revised the manuscript.

DECLARATION OF INTERESTS

K.X., T.L., H.Z., and X.Lai have filed a patent application on the use of MUC1 and α-lactalbumin for anti-coronavirus including SARS-CoV-2 treatment. Q.D. and X.J. have filed a patent application on the use of the SARS-CoV-2 transcomplementation system and its use for anti-SARS-CoV-2 drug screening.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-SARS-CoV-2 spike	Abcam	Cat# ab272504; AB_2847845
Mouse monoclonal anti-β-actin	Proteintech	Cat# 66009-1-Ig; AB_2687938
Goat Anti-Rabbit IgG(H+L)-HRP Conjugated	EASYBIO	Cat# BE0101-100
Goat Anti-Mouse IgG(H&L)-HRP Conjugated	EASYBIO	Cat# BE0102-100
Anti-2019-nCoV S-mIgG1 Antibody (8A5)	Novoprotein	Cat# DA035
Bacterial and virus strains		
SARS-CoV-2 pseudovirus (Luc)	Youchun Wang's Lab (Nie J et al., 2020)	N/A
SARS-CoV-2 pseudovirus (GFP)	Ningshao Xia's Lab (Xiong HL et al., 2020)	N/A
SARS-CoV-2 ΔN trVLP and mutants (GENBANK: MN908947)	Established (Ju et al., 2021; Ren et al., 2021)	N/A
Biological samples		
Human breastmilk	Nanjing Drum Tower Hospital, Nanjing University Medical School, China	N/A
Chemicals, peptides, and recombinant proteins		
α-Lactalbumin	Yuanye Bio	Cat# S12080
Lactoferrin human recombinant, expressed in rice, Iron saturated, >=90% (SDS-PAGE)	Sigma	Cat# L1294
Lactoferrin from human milk	Sigma	Cat# 61327-10MG-F
Lactoferrin from bovine milk	Yuanye Bio	Cat# S24749-1g
Goat Lactoferrin (LTF) Protein	Abbexa	Cat# abx067679
MUC1	Novoprotein	Cat# CS58
MUC4	Cloud-Clone	Cat# RPB034Mu01
Lactadherin/MFGE8	Cusabio	Cat# CSB-EP013752HU
Heparin	HARVEYBIO	Cat# HZB2863-10
Biotinylated Human ACE-2 (C-Avi-6His)	Novoprotein	Cat# CY51
Recombinant 2019-nCoV S-trimer Protein (C-6His)	Novoprotein	Cat# DRA49
Streptavidin/HRP	Solarbio	Cat# SE068
Critical commercial assays		
RNAprep pure cell culture/bacterial total RNA extraction kit	TIANGEN	Cat# DP430
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific	Cat# K1622
Luciferase Assay System	Promega	Cat# E1500
POWRUP SYBR MASTER MIX,5 ML	Life	Cat# A25742
Cell Counting Kit-8 (CCK-8)	DOJINDO	Cat# CK04
Experimental models: Cell lines		
Vero E6 cells	Our lab (Fan et al., 2020)	N/A
Caco-2-N cells	Qiang Ding's Lab	N/A
Huh7.5	Charles M. Rice's Lab	N/A
HEK293T	Charles M. Rice's Lab	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Primers for SARS-CoV-2 RNA Forward: CGAAAGGTAAGATGGAGAGCC Reverse: TGTTGACGTGCCTCTGATAAG	Ju et al. (2021)	N/A
Primers for RPS11 Forward: GCCGAGACTATCTGCACTAC Reverse: ATGTCCAGCCTCAGAACTTC	This paper	N/A
Software and algorithms		
GraphPad Prism version 8	GraphPad Software	v8.0
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
Multiple Sequence Alignment by CLUSTALW	Kyoto University Bioinformatics Center	https://www.genome.jp/tools-bin/clustalw
ESPript 3.0	Robert, X. and Gouet, P. (2014)	https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kuanhui Xiang (kxiang@bjmu.edu.cn)

Materials availability

All materials in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability

Data reported on this paper will be shared by the lead contact upon request. The original sequencing datasets for trVLP of SARS-CoV-2 can be found on the Wuhan-Hu-1, MN908947. The information of trVLP of SARS-CoV-2 is listed in the Key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Collecting and handling of milk samples

The mature breastmilk produced one month after birth in 2017 was collected via pump into sterile containers after disinfecting nipples with 75% ethanol. Samples were frozen in aliquots at −80°C. The skimmed milk samples preparation was performed (Fan et al., 2020). In detail, the mature breastmilk was centrifuged for 15 min at 4,000 × g at 4°C. The upper lipid was discarded and lower aqueous phase was collected and used for further experiments and analysis (Fan et al., 2020). Mothers were informed consent. They were not infected with hepatitis B and C viruses and HIV. This study was approved by the ethics committees of the Medical Center.

Cell lines and key reagents

HEK293T, Vero E6, Huh7.5 and Caco-2-N cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, China) supplemented with 10% fetal bovine serum (FBS) in a humidified 5% (vol/col) CO_2 incubator at 37°C. All cell lines were tested negative for mycoplasma. Goat and cow whey proteins were purchased from Sigma (USA). The recombined lactoferrin (rLF), human lactoferrin (hLF) and bovine lactoferrin (bLF) were purchased from Sigma (USA). Lactadherin, a-lactalbumin were purchased from Sigma (USA). MUC1 encoding from Ala23 to Gly167 is expressed with a Fc tag at the C-terminus and purchased from Novoprotein (China).

Viruses

SARS-CoV-2 pseudovirus with luciferase and GFP expression was kindly shared by Prof. Youchun Wang (National Institutes for Food and Drug Control, China) and Prof. Ningshao Xia (Xiamen University), respectively. The trVLP and their mutants of B.1.1.7 (alpha), B.1.351 (beta), P.1 (gamma) and B.1.617.1 (kappa) expressing GFP replacing viral nucleocapsid gene (N) were used in this study (Ren et al., 2021). Caco-2-N cells were infected with the trVLP to amplify viruses (Ju et al., 2021). P5 to P10 were used in this study.

METHODS DETAILS

The trVLP virus production

The viral production was performed as previously described (Ju et al., 2021). In detail, a full-length SARS-CoV-2 GFP/ΔN cDNA was obtained by digesting the established plasmids with BsaI or BsmBI restriction enzyme (NEB) to get specific sticky end of A, B, C, D and E fragments, which were ligated by T4 DNA ligase. Full-length assembly cDNA was phenol/chloroform extracted and resuspended in nuclease-free water. The viral RNA transcript and viral N mRNA were obtained by *in vitro* transcription with the Mmessage mMACHINE T7 transcription Kit (ThermoFisher Scientific) in 30 μL system. Twenty micrograms of viral RNA and 20 μg N mRNA were mixed in a 4-mm cuvette containing 0.4 mL of Caco-2-N cells (8×10^6 cells) in Opti-MEM medium. The mixture was electroporated by single electrical pulse with a GenePulser apparatus (Bio-Rad) set with 270 V at 950 μF. About three days post electroporation, the produced virus was collected and amplified in Caco-2-N cells for several passages (to P10).

SARS-CoV-2 pseudovirus and trVLP infection assay

Vero E6 or Huh7.5 cells were seeded in a 24-well plate (80,000 cells per well) one day prior to viral infection. The cells were infected with viral inocula of 650 $TCID_{50}$/well. One day post infection (1dpi), the cells were lysed and the luminescence was measured according to the manufacturer's protocol. For trVLP, Caco-2-N cells were seeded in a 24-well plate (80,000 cells/well) one day prior to viral infection and infected with trVLP at multiplicity of infection (MOI) of 0.1. Three days post-infection, the mRNA levels of trVLP and GAPDH were determined by RT-qPCR.

In-gel digestion and LC-MS/MS analysis

Upon SDS-PAGE fractionation, the band of interest was excised and subjected to in-gel trypsin digestion as previously described (Hu et al., 2014). LC-MS analyses of protein digests were carried out on a hybrid ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Velos, Thermo Scientific) coupled with nanoflow reversed-phase liquid chromatography (EASY-nLC 1000, Thermo Scientific). The capillary column (75 μm × 150 mm) with a laser-pulled electrospray tip (Model P-2000, Sutter instruments) was home-packed with 4 μm, 100 Å Magic C18AQ silicabased particles (Michrom BioResources Inc., Auburn, CA) and run at 250 nL/min with the following mobile phases (A: 97% water, 3% acetonitrile, and 0.1% formic acid; B: 80% acetonitrile, 20% water, and 0.1% formic acid). The LC gradient started at 7% B for 3 min and then was linearly increased to 37% in 40 min. Next, the gradient was quickly ramped to 90% in 2 min and stayed there for 10 min. Eluted peptides from the capillary column were electrosprayed directly onto the mass spectrometer for MS and MS/MS analyses in a data dependent acquisition mode. One full MS scan (m/z 400–1200) was acquired by the Orbitrap mass analyzer with R = 60,000 and simultaneously the ten most intense ions were selected for fragmentation under collisioninduced dissociation (CID). Dynamic exclusion was set with repeat duration of 30 s and exclusion duration of 12 s.

Skimmed milk separation

Skimmed milk was precipitated by ammonium sulfate hydrochloride, after centrifugation at 15000×g, 4°C for 2 h, samples were applied to a HiTrap SP HP cation exchange chromatography column (GE Healthcare) equilibrated with buffer A (20 mM Phosphate Buffer, pH7.2). The column was washed with three column vol of buffer A, and bound material was eluted with 10 column vol of buffer B (20 mM Phosphate Buffer 1M NaCl, pH7.2), flow-through and eluted fractions was assayed for testing and identifying the exact fraction with inhibition capacity of SARS-CoV-2 pseudovirus infection. Then, the fraction with capacity to inhibit viral infection was applied to a HiTrap Q HP anion exchange chromatography column (GE Healthcare) equilibrated with buffer A (20 mM Phosphate Buffer, pH7.2). The column was washed with three column vol of buffer A, and bound material was eluted with 10 column vol of buffer B (20 mM Phosphate Buffer 1M NaCl, pH7.2), flow-through and eluted fractions was assayed for testing and identifying the exact fraction with inhibition capacity of SARS-CoV-2 pseudovirus infection. The effective fraction was mixed with 1%

NP-40 and performed to a HiTrap SP HP anion exchange chromatography column (GE Healthcare) equilibrated with buffer A (20 mM Phosphate Buffer, pH7.2). The column was washed with three column vol of buffer A, and bound material was eluted with 10 column vol of buffer B (20 mM Phosphate Buffer 1M NaCl, pH7.2), flow-through and eluted fractions was assayed for testing and identifying the exact fraction with inhibition capacity of SARS-CoV-2 pseudovirus infection. Afterwards, the effective fraction of peak 1 was applied to Superdex 75 10/300 GL size-exclusion chromatography column equilibrated in buffer A, fraction was collected for In-gel digestion and LC-MS/MS analysis.

Viral attachment assay

Caco-2-N, were seed in 24-well plates with 80,000 cells/well one day before the infection. LF (2 mg/mL), MUC1 (2 ug/mL) or α-LA (2 mg/mL) was mixed with trVLP (MOI = 1) at 4°C for 1h, respectively. The mixture was added into the cells and put at 4°C for 2h to allow viral attachment to cells. After washing out of free virus, cell surface trVLP was extracted and quantified by RT-qPCR. For SARS-CoV-2 pseudovirus, Huh7.5 and Vero E6 cells were seed in 96-well plates with 20,000 cells/well one day before infection. LF (2 mg/mL), MUC1 (2 ug/mL) and α-LA (2 mg/mL) was mixed with SARS-CoV-2 pseudovirus (650 $TCID_{50}$/well) at 4°C for 1 h, respectively. The mixture was added into the cells and put at 4°C for 2 h to allow viral attachment to cells. After washing out of free virus, the cells were incubated at 37°C for 24 h. The luciferase assay was performed to detect SARS-CoV-2 pseudovirus infection.

Viral entry assay

Caco-2-N cells were incubated with trVLP (MOI = 1) at 4°C for 1h. Then, the cells were washed with PBS for 3 times. LF (2 mg/mL), MUC1 (2 ug/mL) and α-LA (2 mg/mL) were added into the media and incubated at 37°C for 1h to allow viral internalization into cells, respectively. The mRNA of trVLP was measured by RT-qPCR. GFP were detected under a fluorescence microscope (ECHO laboratories, USA). For SARS-CoV-2 pseudovirus, the method was similar to the trVLP procedure and described as previously described (Fan et al., 2020). In detail, Vero E6 and Huh7.5 cells were seeded in a 24-well plate and then incubated with SARS-CoV-2 pseudovirus (650 $TCID_{50}$/well) at 4°C for 1 h. Then, the cells were washed with PBS for 3 times. LF (2 mg/mL), MUC1 (2 μg/mL) and α-LA (2 mg/mL) were added into the media and incubated at 37°C for 1h to allow viral internalization into cells, respectively. The luciferase assay was performed to detect SARS-CoV-2 pseudovirus infection.

Viral post-entry assay

Caco-2-N cells were infected with trVLP and incubated at 37°C for 1h. After washing out of the free viruses, the cells were cultured in the media containing LF (2 mg/mL), MUC1 (2 ug/mL) and α-LA (2 mg/mL), respectively. Different time point of post-infection, the mRNA of trVLP was measured by RT-qPCR. GFP were detected by a fluorescence microscope (ECHO laboratories, USA). For SARS-CoV-2 pseudovirus, the method was described as previously described (Fan et al., 2020). In detail, Vero E6 and Huh7.5 cells were infected with SARS-CoV-2 pseudovirus (650 $TCID_{50}$/well) and incubated at 37°C for 1h. After washing out of the free viruses, the cells were cultured in the media containing LF (2 mg/mL), MUC1 (2 μg/mL) and α-LA (2 mg/mL), respectively. The luciferase assay was performed to detect SARS-CoV-2 pseudovirus infection.

Viral RNA extraction and quantification

The RNA extraction and quantification were performed as previously described (Ju et al., 2021). Briefly, Total RNA of cells was isolated with the RNAprep pure Cell Culture/Bacterial total RNA extraction kit (Tiangen biotech. Co., China). The cDNA was synthesized by RevertAid first strand cDNA synthesis kit (Invitrogen, USA). The qPCR for SARS-CoV-2 RNA was performed using the 2X RealStar Green Power Mixture (Genstar) according to the instruction. The qPCR primers for viral RNA were as follows: THU-2190 (5′-CGAAAGG TAAGATGGAGAGCC-3′) and THU-2191 (5′-TGTTGACGTGCCTCTGATAAG-3′). GAPDH was used to normalize all the data (Ju et al., 2021).

Western blotting

Western blotting was performed as described previously (Fan et al., 2020). Briefly, cells were lysed with commercial RIPA buffer and centrifuged at 12,000 rpm at 4°C for 30 min to extract the total protein. Cell lysates were loaded on the 12% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane. After blocking with blocking buffer with 5% milk, the membranes were incubated with antibodies. The antibody of anti-SARS-CoV-2 S protein against spike protein and anti-β-actin were used at 1:2000 dilutions. The

second antibody of HRP-conjugated affinipure Goat anti-mouse IgG (H+L) were diluted at 1:20000. SuperSignal® West Femto Maximum Sensitivity Chemiluminescent Substrate (Thermo Scientific, USA) was used for signal development.

Affinity assay between ACE2 and SARS-CoV-2 spike protein

The influence of LF, MUC1 and α-LA on the affinity between ACE2 and SARS-CoV-2 S RBD was performed as previously described (Fan et al., 2020). Briefly, recombinant ACE-2 protein (Novoprotein, China) was immobilized on the MaxiSORP ELISA plate at 100ng per well in 50 μL of 100 mM carbonate-bicarbonate coating buffer over night at 4°C. HRP-conjugated SARS-CoV-2 RBD (Novoprotein, China) at final concentration of 1 ng/μL was mixed with serially diluted human breastmilk. LF, MUC1 and α-LA at different concentrations were added into the ACE-2-coated plate for 1h at room temperature, respectively. The absorbance reading at 450 and 570 nm were acquired using the Cytation 5 microplate reader (Bio Tek).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were analyzed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean ± SD and repeated at least three times (n = 3). Comparisons between the two groups were analyzed using the Student's *t* test. Values of $p < 0.05$ was considered statistically significant, ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.