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Protective effects elicited by cow milk fermented with *L. Paracasei* CBAL74 against SARS-CoV-2 infection in human enterocytes

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**A B S T R A C T**

Fermented foods have been proposed in limiting SARS-CoV-2 infection. Emerging evidence suggest the efficacy of cow’s milk fermented with the probiotic *L. paracasei* CBAL74 (FM-CBAL74) in preventing infectious diseases. We evaluated the protective action of FM-CBAL74 against SARS-CoV-2 infection in human enterocytes. Relevant aspects of SARS-CoV-2 infection were assessed: infectivity, host functional receptor angiotensin-converting enzyme-2 (ACE2), transmembrane protease serine 2 (TMPRSS2), and pro-inflammatory cytokines expression (IL-6, IL-15, IL-1β, VEGF, TNF-α, MCP-1, CXCL1).

Pre-incubation with FM-CBA L74 reduced the number of infected cells. The expression of ACE2 and the pro-inflammatory cytokines IL-6, VEGF, IL-15, IL-1β was downregulated by the pre-treatment with this fermented food. No effect on TMPRSS2, MCP-1, TNF-α and CXCL1 expression was observed.

Modulating the crucial aspects of the infection, the fermented food FM-CBAL74 exerts a preventive action against SARS-CoV-2. These evidence could pave the way to innovative nutritional strategy to mitigate the COVID-19.

1. Introduction

Coronavirus disease 2019 (COVID-19) is an ongoing pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Atzrodt et al., 2020). Gastrointestinal (GI) symptoms (such as diarrhea, nausea, vomiting, anorexia and abdominal pain) are commonly presented by COVID-19 patients, since human enterocytes express high level of angiotensin-converting enzyme-2 (ACE2) receptor, and other cell components for SARS-CoV-2 binding and replication (Berni Canani et al., 2021; Chen, Guo, Pan, & Zhao, 2020; Zang et al., 2020). The spike (S) protein of SARS-CoV-2 binds to the host functional ACE2 receptor through its receptor-binding domain. Then, the activation of S-protein by transmembrane protease serine 2 (TMPRSS2) facilitates viral entry into target cells (Hoffmann et al., 2020; Shang et al., 2020). Viral infection leads to the release of massive amounts of inflammatory cytokines, defined as “cytokine storm” (Lowery, Sariol, Perlman, 2021; Mehta et al., 2020). The cytokine storm attempts to eliminate the infecting virus, but, in the process, collateral damage to human tissue occurs as well (Mangalmurti & Hunter, 2020). Thus, the GI tract is a target organ for SARS-CoV-2 infection, it is the body’s largest immune organ, and exerts a pivotal role in eliciting the cytokine storm in COVID-19 patients (Archer & Kramer, 2020).

Alteration in gut microbiome composition was found in COVID-19 patients, with a reduction of bacteria with relevant immunomodulatory action, such as Faecalibacterium prausnitzii and several bifidobacterial species (Gu et al., 2020; Yeoh et al., 2021; Zuo et al., 2020).
Furthermore, the alteration in gut microbiota reflected disease severity and correlated with the occurrence of cytokine storm in SARS-CoV-2 infected patients (Yeoh et al., 2021). Recent findings ascribed a role of fermented foods in limiting SARS-CoV-2 infection. The consumption of fermented foods has been associated with low death rate induced by COVID-19 in European countries (Bousquet et al., 2021; Fonseca et al., 2020).

In preclinical and clinical studies it has been demonstrated that a specific fermented food product, deriving from the fermentation of cow’s milk with the probiotic L. paracasei CBA L74 (FM-CBAL74), is able to positively modulate several defense mechanisms against pathogens and to prevent GI tract infections in children (Corseillo et al., 2017; Nocerino et al., 2015; Paparo et al. 2017). In addition, the consumption of FM-CBAL74 has been associated with a positive modulation of gut microbiome structure and function (Berni Canani et al., 2017; Roggero et al., 2020).

In this study, we investigated the possible protective action of FM-CBAL74 against SARS-CoV-2 infection in human enterocytes.

Our results revealed that FM-CBL74 reduced the host infection and inflammatory response, thus providing scientific basis for the use of this fermented food against COVID-19.

2. Methods

2.1. Virus

Wild-type (WT) SARS-CoV-2 was isolated from the nasopharyngeal swab of a patient with laboratory-confirmed COVID-19, as previously described (Ferrucci et al., 2021). Briefly, nasopharyngeal swab in 2 ml of viral transport medium was collected for molecular diagnosis and frozen. Confirmed PCR-positive specimen was aliquoted and refrozen until virus isolation was initiated on Vero E6 cells. Vero E6 cells (8 × 10^5) were trypsinized and suspended in DMEM, with 2% FBS in T25 flasks to which 100 μl of the clinical specimen was added. The inoculated cultures were grown in a humidified 37°C incubator with 5% CO₂ and observed for cytopathic effects daily. When cytopathic effects were observed (7 days after infection), the cell monolayers were scrapped with the back of a pipette tip. The cell culture supernatant containing the viral particles was aliquoted (100 μl) and immediately frozen at −80°C. Viral lysates were used for total nucleic acid extraction for confirmatory testing and sequencing. Experiments involving living viruses were performed in accredited biosafety level-3 (BSL-3) laboratory.

2.2. Human enterocytes

We used a well validated model of human enterocytes, the Caco-2 cells (American Type Culture Collection, Middlesex, UK; accession number: HTB-37). This cellular model has been recently used to explore SARS-CoV-2 infection and potential therapeutic strategies against COVID-19 (Chu et al., 2020; Shuai et al., 2020). The cells were cultured in high glucose Dulbecco’s modified Eagle medium (DMEM; Gibco, Berlin, Germany) supplemented with 10% Fetal bovine serum (Sigma-Aldrich; St. Louis, Missouri, USA), 1% Non-Essential amino acids (Sigma-Aldrich; St. Louis, Missouri, USA), 1% (v/v) antibiotics (10,000 U/mL Penicillin and 10 mg/mL Streptomycin) (Euroclone Spa; MI, Italy) and 1% Sodium Pyruvate. Caco-2 cells were grown in an incubator at a temperature of 37°C and CO₂, 5%. The culture medium was changed every 2 days.

2.3. Study products

Study products were provided in powder by Kraft-Heinz Italia, SpA, Latina, Italy an affiliate of Kraft-Heinz Company, co-headquartered in Pittsburgh, PA and Chicago, IL, USA. The fermented milk was prepared from skimmed milk fermented with L. paracasei CBA L74 (FM-CBA L74), (International Depository Accession Number LMG P-24778) which was isolated from the feces of healthy infants. The fermentation was started in the presence of 10⁶ bacteria, reaching 5.9 × 10⁷ colony-forming units/g after 15 h incubation at 37°C. After heating at 85°C for 20 s, in view of inactivating the live bacteria, the formula was spray-dried. The final fermented milk powder contained only bacterial bodies and fermentation products and no living microorganisms. The control (non-fermented milk, NFM) consisted of skimmed milk powder with the same basal probiotic-free nutrients’ composition of fermented milk powder (grams per 100 g): proteins, 35; lipids, 1; carbohydrates, 54.

2.4. Cells infection protocol

Caco-2 cells (2x10⁵ cells/well) were seeded in 6 wells plate and were pretreated for 48 h with 11.5 mg/mL of FM CBA-L74 or NFM. The dose of 11.5 mg/mL has been established based on dose–response experiments and previous data obtained by our group (Paparo et al., 2017). The cells were infected with 0.1 MOI of viral particles that belonged to WT SARS-CoV-2 for 72 h. Subsequently, the cells were washed with PBS, and then harvested in TRIzol or fixed in paraformaldehyde (PFA) for subsequent analysis. Non-infected Caco-2 cells (NI) were used as control. All experiments were carried out in triplicate and repeated three times.

2.5. Infectivity assays: immunofluorescence staining and real time PCR for nucleocapsid protein

To determine the infectivity of SARS-CoV-2, 0.5x10⁵ Caco-2 cells were fixed with 4% PFA (Carlo Erba Reagents, MI, Italy) for 10 min at room temperature and processed for immunofluorescence staining to visualize viral antigen expression (Nucleocapsid protein, N). Auto-fluorescence due to free aldehyde groups from PFA treatment was blocked with 50 mM ammonium chloride (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS for 10 min at room temperature. Cover slips were washed twice with PBS, then cells were permeabilized with 0.1% Triton X-100 (PanReac AppliChem, MI, Italy) in PBS for 10 min. After washing, the cells were blocked for 1 h using 1% BSA in PBS/0.2% Tween 20 and then incubated overnight at 4°C with specific primary antibody for Nucleoprotein (1:100; ProSci, #3851). Coverslips were washed with PBS and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse (1:500; Alexa Fluor 594, #A21203, Invitrogen, MA, USA) for 1 h at room temperature. Nuclei were stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen). Finally, cells were mounted with anti-fading Mowiol (Sigma-Aldrich). Cells were observed with 63x objective on a Zeiss LSM980 confocal system equipped with an ESID detector and controlled by a Zen blue software (Zeiss; Jena, Germany).

To detect N gene in infected Caco-2 cells, total RNA was isolated with TRIzol reagent (Sigma-Aldrich), according to the manufacturer’s instructions. The RNA samples were quantified using the NanoDrop 2000c spectrophotometer (Thermo Scientific) and RNA quality and integrity were assessed with the Experion RNA Standard Sense kit (BioRad, Hercules, CA, USA). RT-PCR for N of SARS-CoV-2 was performed with Lifertiver Novel Coronavirus (2019-nCoV) Real Time kit (BioVendor, Brno, Czech Republic), according to the manufacturer’s protocol. These runs were performed using a 7900 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) under the following conditions: 45°C for 10 min, 95°C for 3 min, then 95°C for 15 s and 58°C for 30 s (×45 cycles). The quantification cycle values of N were reported as means ± SD normalized to the internal control provided by the kit.

2.6. ACE2, TMRPSS2 and inflammatory cytokines analyses by quantitative real time PCR

RNA extracted was reverse transcribed in cDNA with a High-Capacity RNA-to-cDNA™ Kit (Thermofisher, MA, USA) according to the manufacturer’s instructions. Complementary DNA (cDNA) was
stored at −80°C until use.

To evaluate the effect on the ACE2 and TMPRSS2 gene expression, quantitative real-time PCR (qRT-PCR) analysis was performed using Taqman Gene Expression Master Mix (HS0174179_m1 and HS01122322_m1, respectively; Thermofisher).

Inflammatory cytokines expression (IL-6, IL-15, IL-1β, VEGFβ, TNF-α, MCP-1, CXCL1) was evaluated using a SYBR green Master Mix (Applied Biosystems, Grand Island, NY, USA). The details of the primers used in these assays are provided in Table 1. The cDNAs were amplified using a 7900 Real-Time PCR System (Applied Biosystems) at this cycling conditions: 50°C for 2 min, 95°C 2 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by the melt curve setting of 1 cycle of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s.

Data analysis was performed using the comparative threshold cycle (CT) method and expressed as 2−ΔΔCT (25). Beta-glucuronidase (GUSB) gene was used as the housekeeping gene (forward primer: 5′-GAAAATATGTGGTGGAGAGCTCATT-3′; reverse primer: 5′-CCGAGTGAAGATCCCGTTTTA-3′). Expression data were normalized to non-infected cells (N). Each sample was analyzed in triplicate.

2.7. Western blot analysis

Western blotting analysis was carried out following proteins cell extraction by RIPA buffer (50 mM Tris-Hcl, pH 7.6, 150 mM NaCl, 1 mM MgCl2, 1% NP-40) supplemented with a protease and phosphatase inhibitor cocktail. Protein concentrations were estimated using BioRad protein assay dye reagent and BSA (PanReac AppliChem) as standard.

Proteins (30 μg) were separated by SDS-Polyacrylamide gel electrophoresis and subsequently transferred onto Polyvinylidene fluoride (PVDF) membranes (Immobilon™-Transfer Membrane, Tullagreen, Carrigtwohill, Co). Nonspecific protein binding was blocked with a solution containing 5% nonfat dry milk (PanReac AppliChem) and 0.2% Tween20/PBS for 1 h at room temperature.

Primary specific antibodies for ACE2 (1:1000; Abcam, ab15348), and for anti-β actin (1:5000; ACTBD11B7, Santa Cruz, CA, USA) were incubated overnight at 4°C. Sequentially, peroxidase-linked (HRP) conjugated anti-rabbit IgG (1:2000; Abcam, ab205718) and anti-mouse IgG (1:5000; ImmunoReagents, GtxMu-003-DHRPX), respectively, were used as a secondary antibody. Chemiluminescence solution (ECL Wester Antares; Cyanagen) were used for visualizing protein expression. The relative band intensity of each protein was obtained by the normalization to the band intensity of β-actin, using Image Lab Software (Biorad, Hercules, CA, USA).

Table 1 Sequences of the primers used for the analysis of pro-inflammatory cytokines.

| Gene    | Primer    | Primer sequence          |
|---------|-----------|--------------------------|
| IL-6    | Forward   | CGAGAGCGCATCCAGCC       |
|         | Reverse   | GCCCTTTGCGCTTTACAC       |
| IL-15   | Forward   | CAGTTCAGAAGTACACAGATTGA  |
|         | Reverse   | GCCATCOCGGCACTAATGGA     |
| IL-1β   | Forward   | CTTGAAAGGTTATGATGGCTAA   |
|         | Reverse   | GGCCTTACAGGGCCAG        |
| VEGFβ   | Forward   | AGAAGGGAGGACAGGCAATCA    |
|         | Reverse   | GATGGGAGTTGCGTCTG       |
| TNF-α   | Forward   | CTTTCGCGCCGGCCATTTG      |
|         | Reverse   | GATGGAGGAGGTGCCCTG       |
| MCP1    | Forward   | CTATAGAAGGACTCAGAGCGGCG  |
|         | Reverse   | TCTCTTGTGCGCAATGTCG      |
| CXCL1   | Forward   | GGGCCAAACGAGGAGGCTA     |
|         | Reverse   | ATTGGGATGAGGAGGATTGAG     |

IL-6, interleukin-6; IL-15, interleukin-15; IL-1β, interleukin 1-beta; VEGFβ, vascular endothelial growth factor-beta; TNF-α, tumor necrosis factor-alpha; MCP-1, Monocyte Chemoattractant Protein-1; CXCL1, C-X-C Motif Chemokine Ligand 1.

2.8. Statistical analysis

The Kolmogorov-Smirnov test was used to determine whether the variables were normally distributed. Descriptive statistics were reported as the means and standard deviations of 3 independent experiments, each performed in triplicate. The data were analyzed using the one-way ANOVA test. The level of significance for all statistical tests was 2-sided, p < 0.05. All of the data were collected in a dedicated database and analyzed using the GraphPad Prism 7.0 software (La Jolla, CA, USA).

3. Results

The SARS-CoV-2 infectivity was demonstrated by the detection of N viral protein in human enterocytes, using immunofluorescence staining and RT-PCR. Immunofluorescence assay and RT-PCR showed that the pretreatment with FM-CBA L74, but not with NFM, significantly reduced the amount of N viral protein in Caco-2 cells infected with SARS-CoV-2 (Fig. 1A). This result was probably due to a modulation of the SARS-CoV-2 receptor ACE2, the molecular mediator that facilitates virus entry into host cells. In fact, the pretreatment with FM-CBAL74, but not with NFM, resulted in a significant reduction of ACE2 mRNA expression and protein level in human enterocytes (Fig. 1B-C). No effects have been observed on TMPRSS2 expression, suggesting that the preventive action of FM-CBAL74 not involved the modulation of TMPRSS2 (Fig. 1B).

Lastly, we investigated the FM-CBAL74 modulatory action on inflammatory cytokines response elicited by SARS-CoV-2. We evaluated the expression of the most important pro-inflammatory cytokines/chemokines commonly observed in COVID-19 patients (Coperchini, Chiavato, Croce, Magri, & Rotondo, 2020) and we observed that this particular fermented food was able to significantly reduce the expression of IL-6, VEGFβ, IL-15, and IL-1β (Fig. 1D).

4. Discussion

It has been hypothesized that fermented foods could be a potential strategy to limit SARS-CoV-2 infection, but the mechanisms are still largely undefined (Archer & Kramer, 2020). In attempting the use a selected fermented food to limit SARS-CoV-2 infection, it is important to consider a well characterized product. In this study we used a product deriving from a well characterized process in which fermentation of skimmed cow’s milk with the L-lactic acid–producing bacteria isolated from human gut microbiome, L. paracasei CBA L74, is followed by pasteurization to inactivate the microorganism after fermentation. Therefore, the product does not contain living bacteria but the remaining bacterial components, such as bacterial DNA and cell membrane components (e.g. peptidoglycans, polysaccharides, lipotechoic acid), as well as bacterial products produced during the fermentation, such as peptides and metabolites (e.g. lactic acid and other organic acids) (Wegh, Geerlings, Knol, Roeselers, & Belzer, 2019). Lactobacillus paracasei CBA L74 has been deposited in the internationally recognized Belgian collection BCCM / LMG with the International Depository Access Number LMG P-24778 (Proprietary and Confidential information), and is included in the list of “Qualified Presumption of Safety (QPS) microorganism” drawn up by the Panel on Biological Hazards of the European Food Safety Authority (EFSA, 2013). Results from preclinical and clinical studies consistently showed that this product exerts a beneficial action in modulating gut barrier integrity, innate and adaptive immunity, inflammatory cytokines response, microbiome structure and function with a consequent efficient preventive action against GI tract infections (Bern Canani et al., 2017; Corsello et al., 2017; Noceino et al., 2015; Paparo et al., 2017; Roggero et al., 2020; Zagato et al., 2019). In this study, we focused on the infectivity, the expression of the host functional receptor ACE2, TMPRSS2 and the inflammatory cytokines response of SARS-CoV-2 infection in human enterocytes.

We observed that this fermented food was able to significantly reduce the number of infected enterocytes, as demonstrated by the
reduction of N viral protein positive cells. The binding affinity of the S protein of SARS-CoV-2 and ACE2, and its cleaving through the serine protease TMPRSS2, represent the major determinant of SARS-CoV-2 replication rate and disease severity (Hoffmann et al., 2020; Zhou et al., 2020). Blocking of the viral receptor-binding domains represents a key step in antiviral approaches (Huang, Yang, Xu, Xu, & Liu, 2020). We found that FM-CBA L74 was able to reduce ACE2 expression, preventing SARS-CoV-2 entry into human enterocytes. No modulation was observed in TMPRSS2 expression.

One of the characteristics of COVID-19 is the exacerbated inflammatory response that is considered an important determinant of clinical severity and even death in COVID-19 patients (Huang et al., 2020; Yang et al., 2020; Buszko et al., 2021). The GI tract contributes to the genesis of the cytokine storm (Archer & Kramer, 2020). Furthermore, alteration of gut microbiome plays a role in modulating host inflammatory response. The depletion of several bacterial species in the COVID-19 patients correlated with an increased plasma concentration of several cytokines, chemokines and inflammatory biomarkers, such as IL-1β and IL-6 (Yeoh et al., 2021).

The modulation of these cytokines is considered a relevant target to improve COVID-19 outcomes (Qian et al., 2021). In our study, we observed that FM-CBAL74 was able to reduce the transcripts of IL-1β, IL-6, VEGFβ, and IL-15. The result on IL-6 could be particularly relevant in clinical practice. Increased IL-6 levels are commonly observed in patients with COVID-19 with an association with disease severity (Coomes & Haghybayan, 2020). Accordingly, clinical trials are providing promising results on the effects of IL-6 inhibitors in COVID-19 patients (Khan et al., 2020; Roumier, Paule, Groh, Vallée, & Ackermann, 2020).

The research on active compounds able to counteract SARS-CoV-2 infection is very active (Peter, Sandeep, Rao, & Kalpana, 2021). We have recently provided evidence on phosphate foods additives, including long chain PolyP that at low dose are able to inhibit SARS-CoV-2 infection, replication and cytokine storm (Ferrucci et al., 2021). The results of the present study provide additional evidence on the potential of nutritional strategies against COVID-19.

The limitations of this study were that we didn’t explore other molecular mechanisms involved in SARS-CoV-2 infection, and which specific component of this fermented food could be responsible for the observed protective actions. The effects could be related to the activities of several components, including lipoteichoic acid, peptidoglycans, bacteriocins, nucleotides and peptides. In particular, it has been demonstrated that peptides deriving from fermentation of cow’s milk proteins could act as modulators of non-immune and immune GI defense mechanisms (Hering et al., 2011; Marshall, 2004; Playford, Macdonald, & Johnson, 2000; Vinderola, Matar, Palacios, & Perdiguén, 2006).

In conclusion, we provided evidence on the protective action elicited by FM-CBAL74 against COVID-19. Our findings could act in parallel with other beneficial actions on gut microbiome structure and function, and on innate and adaptive immunity that have been highlighted in previous studies (Berni Canani et al., 2017; Corsello et al., 2017; Nocerino et al., 2015; Paparo et al., 2017; Roggero et al., 2020). Altogether, these data suggest the potential of this innovative nutritional strategy to mitigate COVID-19.

5. Ethics statement

The study not involves humans and animals, but it is an in vitro study.

6. Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Lorella Paparo: Conceptualization, Data curation, Methodology, Software, Validation, Writing – original draft, Writing - review & editing. Cristina Bruno: Methodology, Software, Supervision. Veronica Ferrucci: Investigation, Supervision,Validation. Erika Punzo: Investigation, Methodology, Validation. Maurizio Viscardi: Investigation, Methodology, Validation. Pellegrino Cerino: Investigation, Methodology, Validation. Alessia Romano: Formal analysis, Software, Investigation, Validation. Massimo Zollo: Conceptualization, Funding acquisition, Validation, Writing – original draft, Writing - review & editing. Roberto Berni Canani: Conceptualization, Data curation, Funding acquisition, Validation, Supervision, Project administration, Writing – original draft, Writing - review & editing.

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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The paper is dedicated to all patients who died because COVID-19 at our Center during this pandemic.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104787.

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