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Functional foods for infectious diseases: protective action elicited by a postbiotic derived from cow milk fermentation with *Lacticaseibacillus paracasei* CBAL74 against *Rotavirus* and SARS-CoV-2

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Brief thesis presentation

Emerging evidence suggest the efficacy of the postbiotic derived from cow's milk fermentation with the probiotic *Lacticaseibacillus paracasei* CBA L74 (FM-CBAL74) in preventing pediatric infectious diseases.

Rotavirus (RV) is a non-enveloped double-stranded RNA (dsRNA) virus that is the leading cause of acute gastroenteritis-associated morbidity and mortality in early childhood. RV is responsible >500.000 deaths and approximately 2.4 million hospitalization in young children worldwide annually.

SARS-CoV-2 is a single-stranded RNA (ssRNA) virus responsible for the Coronavirus disease (COVID-19) pandemic responsible for around 5.6 million deaths and more than 110 million cases reported globally in the last 2 years.

Here, we investigated the protective action elicited by FM-CBAL74 against RV and SARS-CoV-2 infection in an *in vitro* model of human gut epithelium.

We demonstrated that pre-incubation with FM-CBAL74 inhibited RV-induced epithelial barrier damage (tight junctions (TJs) and adherent junctions' protein (AJ), and transepithelial electrical resistance evaluation), reactive oxygen species (ROS) and pro-inflammatory cytokines (IL-6, IL-8, TNF-α) release, through MAP kinases pathway activation.

Regarding SARS-CoV-2 infection, we demonstrated that pre- incubation with FM-CBA L74 reduced the number of infected cells, the expression of the host functional receptor angiotensin-converting enzyme-2 (ACE2) and the pro-inflammatory cytokines IL-6, VEGFB, IL-15, IL-1β release. Modulating immune and non-immune protective mechanisms, the postbiotic FM-CBAL74 exerts a protective action against RV and SARS-CoV-2 infection. These findings may open new preventive and therapeutic strategies for these conditions.

Riassunto

Sempre nuove evidenze suggeriscono l'efficacia del postbiotico derivato dalla fermentazione del latte vaccino con il probiotico *Lacticaseibacillus paracasei* CBA L74 (FM-CBAL74) per la prevenzione delle malattie infettive del bambino.

Il *Rotavirus* (RV) è un virus a RNA, a doppio filamento, ed è la principale causa di morbilità e mortalità associata alla gastroenterite acuta nella prima infanzia. Ogni anno è responsabile di oltre 500.000 decessi e di circa 2,4 milioni di ricoveri ospedalieri in bambini piccoli in tutto il mondo.

SARS-CoV-2 è un virus a RNA, a singolo filamento, responsabile della pandemia di Coronavirus (COVID-19), responsabile di oltre 5.6 milioni di morti e 110 milioni di casi a livello globale negli ultimi 2 anni.

In questo progetto di Dottorato, abbiamo studiato l'azione protettiva elicitata da FM-CBAL74 contro l'infezione da RV e SARS-CoV-2 in un modello *in vitro* di epitelio intestinale umano.

Abbiamo dimostrato che la pre-incubazione con FM-CBAL74 inibisce il danno di barriera epiteliale indotto da RV (proteine delle giunzioni serrate e aderenti, resistenza elettrica transepiteliale), lo stress ossidativo ed il rilascio di citochine pro-infiammatorie (IL-6, IL-8, TNF-α) attraverso l'attivazione della via delle MAP chinasi.

Nell'infezione da SARS-CoV-2 abbiamo dimostrato che la pre-incubazione con FM-CBA L74 riduce il numero di cellule infette, l'espressione del recettore ACE2 (enzima 2 di conversione dell'angiotensina), il rilascio di citochine pro-infiammatorie IL-6, VEGFB, IL-15, IL-1β.

In conclusione, il postbiotico FM-CBAL7, attraverso una modulazione di meccanismi di difesa immunologici e non-immunologici, esercita un'efficace azione protettiva dei riguardi dell'infezione da RV e SARS-CoV-2. Questi dati supportano un utilizzo clinico di questo postbiotico per la prevenzione ed il trattamento di queste due temibili infezioni.

1.0 INTRODUCTION

1.1 The postbiotic concept

Postbiotics are defined as *"any factor resulting from the metabolic activity of a probiotic or any released molecule capable of conferring beneficial effects to the host in a direct or indirect way"* [1]. The nature and mixture of postbiotics depend on the strain of probiotic and on the matrix used for fermentation. Most of the beneficial activities of probiotics are mediated by postbiotics. Hence, postbiotics may substitute for probiotics by directly providing the active components, which can mediate their effects on human cells and microbiome [2].

The effects could be related to the activities of several components, including lipoteichoic acid, peptidoglycans, bacteriocins, short-chain fatty acids (SCFAs), nucleotides and/or peptides that are generated in the fermented matrix [3].

It has been demonstrated that the postbiotics have an immunomodulatory, anti-inflammatory, and antimicrobial effect. To fully understand the postbiotic's mode of action, it is essential to understand the intestinal surface structure and the layers in contact with gut bacteria [4]. The intestinal epithelium provides the first physical barrier against microorganisms in the gut lumen [5] and protect the host from the environment. This barrier comprises the mucus layer produced by the goblet cells, followed by a monolayer of epithelial cells forming the epithelial junction adhesion complex. Once this barrier function is disrupted, bacterial and food antigens can reach the submucosa and induce inflammatory responses [6]. The intestinal barrier's protective effect can be exerted by increasing intestinal mucin (MUC2) production and the expression of genes involved in tight junction (TJ) signaling (such as OCLN, CLDN-4 and ZO-1) [7]. In addition to the ability to reduce inflammation modulating IgA's immunity and production [4]. All the above, reinforcing intestinal barrier integrity to maintain the intestinal homeostasis [8].

Postbiotics represent a safe method to improve host health; the absence of viable microorganisms minimizes the risks associated with their intake. A safety advantage was that they decrease the risk for microbial translocation from the gut lumen to blood, of infections and prevent potential detrimental effects shown by probiotics within a pro-inflammatory context [9]. They can bypass the problem of acquiring antibiotic resistance genes and virulence factors, which may occur when probiotics are used [10].

Postbiotics eliminate the need for exposure to live microorganisms, which is particularly important in children with an innate and adaptive immature immune system [11], and who may still acquire viral, bacterial, and parasitic infections. Furthermore, other important factor driving interest in postbiotics is their inherent stability, both during industrial processes and storage. Many probiotic organisms are sensitive to oxygen and heat; and could be damaged in those geographical regions that do not have reliable cold chains or whose ambient temperature causes problems for storage of live microorganisms [12].

1.2 The postbiotic derived from cow milk fermentation with the probiotic *L.paracasei* CBA L74

Fermentation process has been one of the oldest methods of food and beverage preservation and of improving the functionality, health-promoting properties, and nutritional of food products [13]. This process is a primarily an anaerobic process, used for thousands of years, to convert sugars, such as glucose, to other compounds like alcohol, while producing energy for the microorganism or cell [14].

Archaeologists have found molecular evidence for the production of a fermented beverage dated back to 7000 BC from the Neolithic village of Jiahu in China [15]. The earliest evidence of wine production, making comes from the presence of tartaric acid in an ancient jar dated from 5400–5000

BC, was found at the Hajji Firuz Tepe site in the northern Zagros Mountains in Mesopotamia [16].

The first traces of fermented milk products have been found as early as 8000 B.C. in Asia Minor and Eastern Europe, soon after the domestication of milk-producing animals (cows, sheep, and goats)[17]. In the 1850s and 1860s, Louis Pasteur became the first scientist to study fermentation, demonstrating that this process was performed by living cells [18]. According to Pasteur, only microorganisms could convert sugars into alcohol from grape juice, and that the process occurred in the absence of oxygen. He defined fermentation as *"respiration without air"* [14].

In 1905, a Bulgarian medical student, Stamen Grigorov discovered *L.delbrueckii subspecies bulgaricus* (known until 1984 as *L.bulgaricus*), the lactic acid bacterium responsible for processing milk into yoghurt [19]. Based on Grigorov's findings, in 1909 the Russian biologist and Nobel Prize Elie Metchnikoff, proposed that daily yoghurt consumption engenders the longevity of the Bulgarian peasant population. Suggesting in this way a connection between the consumption of yoghurt and the number of Bulgarian centenarians [20].

Lactic acid bacteria (LAB) have been used for centuries in the foods and beverages industries and plays nowadays a significant role in in numerous fermentation processes [21]. They used in food preservation and for the modification of the organoleptic characteristics of foods, for example flavors and texture. The general description of the bacteria included in the group is Gram-positive, non-spore forming, aerotolerant bacteria, catalase negative. Shape is variable, from cocci through to elongated rods [22]. Most LAB are non-motile, and may grow in high salt concentrations, and at a low pH. Lactic acid bacteria are phylogenetically classified in the phylum *Firmicutes*, class *Bacilli*, and order *Lactobacillales* [23]. The order *Lactobacillales* currently includes six families, i.e., *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae*, and *Streptococcaceae*, with 40 genera and a continually increasing number of species (>400) [22]. The genus *Bifidobacterium* (family *Bifidobacteriaceae*) is also included in the LAB group, although it belongs to the phylum Actinobacteria. These bacteria may have common features with LAB

(mostly the ability to produce lactic acid) and are called "LAB-related" [24].

Lactic acid bacteria can be classified as homofermentative and heterofermentative. Homofermentative bacteria produce lactic acid (LA) as the key carbohydrate fermentation products [25], while heterofermentative bacteria additionally produce other products such as acetic acid, CO2, ethanol, acetoin, and diacetyl [26].

Some LAB improves the nutritional quality of fermented foods by increasing vitamins, minerals, dietary fiber, and antioxidants [27]. Furthermore, some LAB can produce exopolysaccharides, aroma compounds, and important enzymes, which plays a significant role for sensory quality of fermented foods [28].

Lactic acid bacteria are predominantly found in nutrient-rich habitats. They are part of the normal microbiota of the gastrointestinal tract (GIT) and of the vagina of animals and humans, and they constitute an important element of the non-starter microbial communities found in dairy products (e.g., milk, cheeses, kefir), fish, meat, and vegetables. They are isolated from various food matrices and used as probiotics; due to their health benefits[29]. They are, in fact, proficient in inhibiting the growth of pathogenic organisms through different mechanisms such as adherence to epithelial cells, withstanding low pH, gastric juice, modulation of the immune system, and secretion of antimicrobial compounds [30].

Furthermore, they enjoy historical “generally regarded as safe” (GRAS) and “qualified presumption of safety” (QPS) status by the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA), respectively.

These bacteria, moreover, can be used as starter culture in the fermentation process under controlled conditions [25].

In this study we focused on the probiotic *L.paracasei* CBA L74, a Gram-positive

homofermentative, facultative anaerobic bacteria 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). This bacterial strain of human origin has been isolated from healthy infants' microbiota and it has been genetically characterized by the identification of its molecular profile using the Rep-PCR (Repetitive Extra-Genic Palindromic-PCR).

Any food product amenable to fermentation by *L.paracasei* CBA L74 may be used, but the fermentation of cow milk with this probiotic has been proved to be a successful route to produce functional foods. Several studies reported both in preclinical and clinical evidence on the health benefits elicited by anew postbiotic deriving from cow milk fermentation with the probiotic *L.paracasei* CBA L74 [2,31-35].

These studies have shown that this postbiotic stimulates innate and adaptative immunity, modulates inflammation and gut microbiome structure and function and protects against pediatric common infectious diseases [2,31-35].

1.3 Preparation process of the postbiotic

The postbiotic investigated in the project thesis derived from skimmed cow milk fermentation with the probiotic *L.paracasei* CBA L74 (International Depository Accession Number LMG P-24778). The 4-day preparation process consisted of 3 phases fermentation, inactivation and drying, all performed at "Heinz Innovation Centre" in Nijmegen, The Netherlands (Figure 1). A figurative scheme of the plant is reported in Figure 2.

Fermentation was started in the presence of 10^6 bacteria for 24h at 37 °C, reaching 5.9 x 10^9 colony-forming units/g. The recipe for fermentation was 10.5% (w/v) skimmed powder cow milk, 87.5% (w/v) water and 2% (w/v) dextrose (Table 1). Before starting the fermentation, a preliminary phase was necessary for sterilization and preparation of the equipment (some steps must be done in the

laboratory, others in the plant); then the fermentation and inactivation phase took place in sequence. The detailed protocol is reported below and is summarized in Figure 3.

Preliminary preparation phase:

- pre-inoculum preparation (10 ml) and incubation for 24h at 30 °C (A.2) (Day 1)
- inoculum preparation 2L and incubation for 24h at 30 °C (A.2) (Day 2)
- inoculum valve and glassware sterilization in autoclave (Day 2)
- 4 L of NaOH 30% w/v (7.5M) preparation (Day 2)
- weighing of all the ingredients to use to ferment (Day 3)
- calibration of the probe for pH/ temperature measurements (Day 3)
- check of the air filter (Day 3)
- cleaning of the line (with cleaning solutions) (Day 3)
- sterilization of the UHT line (Day 3)
- de-aeration of the fermenter (Day 3)
- connection and steam flushing of the inoculum and sodium hydroxide valves (Day 3)
- sterilization of the fermenter with steam at 121 °C for 30 minutes (Day 3)

Fermentation and inactivation phase: (Day 3)

- Ingredients mixing and holding for 20 min (powder milk, water, dextrose)
- 1st direct steam injection at 85-90 °C for 10 seconds
- 2nd direct steam injection at 137 °C for 10 seconds
- Fermentation substrate cooling (the cooling takes place in a tube heat exchanger in which the exterior tube is provided by water at 20 °C)
- Filling of fermenter
- Fermentation substrate at 37 °C
- Transfer of the inoculum into the fermenter

- ❖ Fermentation is carried out for 24 h (samples are taken every 2h; night shifts are necessary)
- ❖ Inactivation phase is carried out bringing the system at 85°C and holding of 20 seconds (setting the temperature of the fluid in the outer jacket of the bioreactor at 90°C) (Day 4)

On the fourth day, spray drying was also carried out. It was a vertical axis spray dryer with a co-current flow and two fluid nozzle atomization. The final fermented milk powder contained only bacterial bodies and fermentation products and no living microorganisms.

1.4 Preclinical evidence on the preventive action against pediatric common infectious diseases

Gut barrier represents the first mechanism of defense from the external environment [5]. Disruption of gut mucosal integrity results in increased permeability to allergens, toxins and pathogens, leading to immunological stress response and inflammation. Increased permeability to luminal toxins, allergens and pathogens plays a crucial role in the pathogenesis of a number of gastrointestinal diseases [36]. The integrity of gut barrier is so essential for the maintenance of proper intestinal homeostasis and efficient protective reactions against chemical and microbial challenges.

The first layer of defense in the epithelium of the gut is formed by the mucus layer which functioning as a dynamic protective barrier [37]. The intestinal mucus layer is principally comprised mucins, glycoproteins, lipids, secretory immunoglobulin (Ig) A and antimicrobial peptides (AMPs) such as α-defensins, that controls the passage of molecules and prevents pathogenic bacteria, viruses, and parasites from reaching the epithelial barrier [38].

The mucus layer of the gut is produced and maintained by goblet cells that continuously renewed it every 1–2 hours [39]. In the large intestine, the mucus is organized in two different layers: the inner and the outer layer. Although it was observed that they have almost identical protein profiles, there are significant differences between them. The inner mucus layer is continuously refilled by the

MUC2 mucin, is anchored to the goblet cells and remains attached to the epithelium. It has a stratified appearance due to its organization in flat sheets, placed one below the other and forming a lamellar inner mucus layer. The outer mucus layer expands four times in volume, maintaining the netlike structure and avoiding the dissolution of the mucus gel due to the disulfide bonds. Intestinal infections induced by parasites, viruses, and bacteria cold influence the production of mucin by goblet cells [40].

Tight junction (TJ) proteins form a continuous intercellular network creating a barrier with selective regulation of water, ion, and solutes across endothelial, epithelial, and glial tissues [41]. Tight junction (TJ) structures formed by proteins, such as occludin (OCLN), claudin (CLDN), zonula-occludens (ZO) play a key role in intestinal barrier function. These transmembrane proteins are programmed to rapidly open and close the barrier, in response to various stimuli modulating paracellular permeability [42]. Disturbances in TJ barrier function result in increased paracellular permeability and increased exposure of underlying intestinal tissue and other organ systems to noxious luminal antigens [43].

Xiang Ling et al. [44], showed the protective effect of Bifidobacterium on intestinal barrier function both in LPS-induced enterocyte barrier injury of Caco-2 monolayers and in a necrotizing enterocolitis (NEC) rat model. The molecular mechanisms associated with these protective effects include inhibition of proinflammatory cytokine secretion, suppression of zonulin protein release and improvement of intestinal TJ integrity. Importantly, zonulin may be a crucial factor by which Bifidobacterium protects intestinal barrier function [44].

Zagato et al. [31], found that bacterial culture medium or milk fermented with FM-CBAL74 has strong anti-inflammatory properties on dendritic cells in response to the inflammatory enteric pathogen *Salmonella* typhimurium. Furthermore, they demonstrated that FM-CBAL74 has protective effects against colitis when administered intragastrically.

Paparo et al. [34], demonstrated that FM-CBAL74, through a direct interaction with human enterocytes, can regulate the integrity of gut mucosa and immune factors. This effect has been evaluated on human enterocytes cell lines (the Caco-2 cells), stimulated for 48 hours at different concentrations. The positive modulation of gut mucosa integrity by FM-CBAL74 has been supported by the up-regulated expression of TJ proteins, a significant increase of MUC2 expression and mucus layer thickness, and an up-regulation of innate immunity peptides (HBD-2 and LL-37).

Toll-like receptors (TLRs) as pattern recognition receptors (PRRs) can activate innate immunity, via sensing invasion of microbial pathogens [45]. Paparo et al. [34], shown an increase in TLR2, NF-kB1 and TFF3 expression after FM-CBAL74 exposure. Furthermore, they found a down-regulation of TLR4 expression, further supporting a potential anti-inflammatory activity of FM-CBAL74, as previously demonstrated in human dendritic cells [31].

1.5 Clinical data on preventive action against pediatric common infectious diseases

Common gastrointestinal and respiratory tract infections continue to be an important cause of morbidity for young children aged 0–3 years, especially during the winter season [46].

Daycare centers (DCCs) worldwide are ideal places for infections to spread because of the density of small children and their constant interaction [47]. Common infectious diseases (CIDs) are ease by a general immaturity of the immune system and of respiratory and gastrointestinal tract functions [33]. This condition makes infants and children more susceptible to various infectious pathogens [48]. Children attending DCCs, in fact, are at increased risk for acquiring acute infections which is 2–3 times higher compared to children who stay at home or in small family care groups [32,49].

The frequency of these infections causes significant discomfort for children and their parents, as well as, high rates of medical examinations, use of drugs, need for hospitalization, and many missed days of both daycare and parental work [33]. Parents of children attending child-care facilities miss

from 1 to 4 weeks of work per year caring for ill children [50].

Illnesses related to daycare centers have been estimated to cost $1.8 billion per year in the United States [51].

Respiratory tract and enteric pathogens are the most commonly infectious agents associated with disease outbreaks in daycare centers [50]. It is estimated that about 25% of children under 1 year old and 6% of children during the first 6 years of life have recurrent respiratory infections (RRIs), making them one of the most common reasons for pediatric medical visits in the early years of life [52]. Enteric viruses, especially *Rotavirus* (RV), instead, have been recognized as the leading cause of childhood diarrhea worldwide [53]. Children of any age may be affected by RV, but attack rates are highest for children less than 5 years of age with the peak incidence as occurring between 6 and 24 months of age [54]. All these pathogens may be transmitted directly from child to child via sneezing, coughing, and touching, or indirectly via the environment [55].

Understanding the epidemiology of infectious illnesses, can assist in predicting transmission within a childcare facility [56].

It would be important to find a reliable strategy for the prevention of CIDs in children. An option is to use postbiotics.

Two randomized controlled trials (RCTs) have been performed to explore the effects of FM-CBAL74 in the prevention of gastrointestinal and respiratory tract infections in children who attend day care centers. In the first RCT [32], healthy children (12–48 months of age) attending daycare or preschool at least five days a week, were invited to participate to the study and distributed, randomly, into three groups (A, B and C) of 3-month dietary treatment during the winter season: postbiotic deriving from the fermentation of cow milk with the probiotic FM-CBAL74 (group A), postbiotic deriving from the fermentation of rice with the probiotic FM-CBAL74 (group B), placebo (group C). All subjects received 7 g/day of study products diluted in a maximum 150 ml of

cow's milk or water. After dilution, the look and the taste were the same for all study products.

The primary outcome of the trial was the proportion of children experiencing at least one episode of CID. The secondary outcomes were the proportion of children with recurrent CID (i.e., ≥ 3 episodes), total number of CIDs, use of medications (antipyretics, antibiotics, or steroids), emergency department visits, pediatric visits by the family pediatricians (FP) and hospitalizations. Study groups were also compared for fecal levels of α- and β-defensins, cathelicidin (LL-37), secretory immunoglobulin A (sIgA) at enrollment and after 3 months of intervention.

The overall number of children presenting at least one episode of CID, the total number of CIDs and their number per child revealed statistically significant differences in favor of the treated groups. This protective effect was accompanied by a reduction of drugs use. Intention-to-treat analysis showed that the proportion of children who experienced at least one CID was lower in group A (51.8%) and B (65.9%) compared to group C (80.3%). Per-protocol analysis showed that the proportion of children presenting upper respiratory tract infections was lower in group A (48.2%) and group B (58.5%) compared with group C (70.5%). The proportion of children presenting acute gastroenteritis was also lower in group A (13.1%) and group B (19.5%) compared with group C (31.1%). Furthermore, was observed for groups A and B compared to group C, an immunostimulatory effect consisting in a significant increase in innate (1-3: α- and β-defensins and cathelicidin (LL-37)) and acquired (secretory immunoglobulin A (sIgA)) immunity peptides production.

The study provided evidence that fermented foods were effective in protecting children attending daycare or preschool against CIDs, with a higher protective effect in children who received a daily supplement with postbiotic deriving from fermentation of cow milk with FM-CBAL74.

Interestingly, these results have been confirmed by another multicenter randomized and controlled trial with a similar study design [33]. Healthy children (12–48 months of age) attending daycare or

preschool for at least 5 days a week, were allocated randomly to two groups and supplemented daily for 3 months with cow's skim milk fermented with FM-CBAL74 (group A) or placebo (group B) [33]. All subjects received 7 g/day of study products diluted in a maximum 150 ml of cow's milk or water. After dilution, the look and the taste were the same for all study products.

The primary outcome of the trial was the rate of children experiencing at least one episode of CID. The secondary outcomes were total number of CIDs, use of medications (antipyretics, antibiotics, steroids), emergency department medical examinations, hospitalizations, days of work lost by the parents, and days of school lost by children. Study groups were also compared for fecal levels of 1-3: α- and β-defensins, cathelicidin (LL-37), and secretory immunoglobulin A (sIgA) at enrollment and after 3 months of intervention.

The results of this study confirmed that dietary supplementation with cow's skim milk powder fermented with FM-CBAL74 is associated with a reduction of CIDs in young children attending school in addition to a reduction of medication use, working days, or days of school lost, and to a significant increase in innate and acquired immunity peptides production. The dietary supplementation was well accepted by the children and safe, as demonstrated by the low dropout rate together with the high level of adherence, and the absence of adverse events observed during the study period.

Following this, cow milk fermented with FM-CBAL74 may be used as a valid strategy in preventing CIDs in children attending educational programs.

On the other hand, it is important to recognize that these RCTs studied a specific fermented product with a specific probiotic strain, a well-defined dose, and age-group, and that these findings cannot be extrapolated for other fermented products based on different probiotic strains.

1.6 Modulatory action on human gut microbiome

The human gastrointestinal tract (GIT) is one of the most complex ecosystems known, colonized by a vast variety of microbial population. Its microbiota consists of 100 trillion bacterial cells (10-fold more than the total number of human cells), and an average of 1200 different species [57].

Bacteria represent the most abundant colonizers, but archaea, fungi, eukaryotic microbes and viruses are also abundant in healthy individuals [58].

Everyone has a unique and specific gut microbial composition like a peculiar *fingerprint,* and it hasbeen agreed that the development of different types of diseases in humans could be related to the disruption of the gut microbiome, such a condition known as *dysbiosis* [59]. Dysbiosis refers to an imbalance in microbial species abundance, which is commonly linked to impaired gut barrier function and inflammatory cell activation [60,61]. Hence, maintaining a healthy proportion of beneficial microbes, also called *eubiosis*, is essential for human health [62].

Numerous studies have demonstrated that human gut microbial colonization begins at birth but continues to develop a succession of taxonomic abundances for two to three years until the gut microbiota reaches adult-like diversity and proportions [63]. Several factors, including gestational age (GA), delivery mode, birth weight, antibiotic exposure, maternal microbiome, and diet, could modulate the diversity, abundance, and function of early life gut microbiota [63]. For example, infants born by cesarean section exhibit decreased diversity and richness of the gut microbiota, as a decreased *Bifidobacterium, Bacteroidetes, and Lactobacillaceae* [64]. In addition, recent evidence has demonstrated elevated colonization of opportunistic pathogens, many containing antibiotic resistance genes, in infants born by cesarean section [65]. Other studies have shown a link between caesarean-section delivery and the development of asthma, allergic rhinitis, and eczema [66,67].

The microbiota offers many benefits in host defense against pathogens, through a range of physiological functions such as strengthening gut integrity or shaping the intestinal epithelium [68].

It also performs an essential metabolic function, acting as a source of essential nutrients and

vitamins and aiding in the extraction of energy and nutrients, such as short-chain fatty acids (SCFA) and amino acids, from food [69].

The principal system we have for interacting with the microbiota is the immune system. It known, in fact, a mutualistic interaction occurring between gut microbiota and immune system, where gut microbiota influences immune system development and function, and the immune system shapes gut microbiota composition [35].

Bifidobacterium and LAB have been shown to secrete factors that hinder inflammation, presumably via the downregulation of interleukin-8 secretion, NF-kB dependent gene expression, and macrophage-attracting chemokine production. Some studies suggest that microbial-derived SCFAs, acetate, propionate, and butyrate, may be contributing to the modulation of host immune responses directly via G-protein-coupled receptors (GPCRs), such as GPR43 and GPR41, and by epigenetic mechanisms, such as methylation activity within the promoter regions of certain genes [70].

In Berni Canani et al. [35], has been reported the effect of cow milk fermented with FM-CBAL74 on the gut microbiota composition and butyrate production. Healthy children (12 to 48 months of age) attending daycare or preschool at least 5 days a week, were randomly selected from the previous RCT [33], and invited to participate to the study. Subjects were supplemented daily for 3 months with either a dietary product deriving from cow's milk fermented with *L.paracasei* CBA L74 or a placebo.

Fecal microbiota was profiled using 16S rRNA gene amplicon sequencing, and the fecal butyrate concentration was also measured. In this study was observed that the treatment with FM-CBAL74 affected gut microbiota with significant differences between the two study groups following intervention. The relative proportion of *Lactobacillaceae* and *Ruminococcaceae* significantly increased following FM-CBAL74 treatment, with specific significant increases in *Oscillospira* and *Faecalibacterium.* These results found positive correlations between the relative abundance of

several genera belonging to *Ruminococcaceae* and fecal LL-37 level, whereas *Lachnospira* and *Ruminococcus (Lachnospiraceae)* correlated with HBD-2 levels. Furthermore, FM-CBAL74 treatment resulted in an increase in the relative abundance of genes involved in butyrate synthesis, especially genes encoding butyryl-CoA transferase (EC 2.8.3.8) and butyrate kinase (EC 2.7.2.7). Consistently, a significant increase in fecal butyrate levels in children consumed FM-CBAL74 was observed; likely deriving from lactate catabolism, one of the primary pathways for butyrate production by gut bacteria [35].

In conclusion, this study has shown that FM-CBAL74 induces positive regulation of the mutual interaction between the immune system and gut microbiota.

Additionally, the postbiotic deriving from fermentation of cow's milk with FM-CBAL74 has been shown to be an effective substitute dietary strategy, when breast milk is not available, or it is not sufficient to satisfy the nutritional needs.

In a monocentric, randomized, and controlled trial [2], healthy full-term infants were screened for participation in the study, to evaluate the effects of two different dietary regimens on immune defense mechanisms (primary endpoint: secretory IgA, antimicrobial peptides), the microbiota and its metabolome (secondary outcomes).

Newborns were randomized to receive until the third month of age-standard formula containing 2.3 g/100 g of FM-CBAL74 powder (formula F group) or standard formula (formula S group), which is equivalent to 0.3% in the ready to use infant formula. The reference group was constituted only by breastfed infants. The results obtained in this RCT shown that the fermented formula, safe and well tolerated, induced an increase in secretory IgA (but not in antimicrobial peptides), reaching values similar to those of the reference group. On the contrary, formula S-fed infants displayed little increase. Overall, these data indicate that a fermented formula can drive sIgA production by the newborns.

The fermented formula, also, reduced the diversity of the microbiota, similarly, but not as much as, breastmilk. In fact, vaginally delivered and formula F-fed infants had a more homogeneous microbiota, those fed formula S had a more heterogeneous microbiota.

Furthermore, has been found that formula-fed infants demonstrated a differentiation in fecal metabolites compared with breastfed infants. In particular, the metabolome of breastfed infants was characterized not only by monosaccharides, such as sorbose and rhamnose, but also by fatty acids such as dodecanoic acid. This is likely due to metabolites contained within the breast milk that are transferred to the newborns. Although fecal metabolites of formula-fed infants were still different compared with those of breastfed infants, the metabolome of formula F-fed infants was closer to that of the reference group. Specifically, in fecal samples of formula S-fed infants, has been observed a higher amount of carboxylic acids, such as fumaric and acetic acid, and saturated fatty acids such as myristic acid, than in breastfed and formula F-fed infants.

Together, all these results suggest that a fermented formula favors the maturation of the immune system, microbiota, and metabolome.

2.0 Aims of the study

The idea of this PhD thesis research project derived from all the previously reported data, suggesting a protective action elicited by the postbiotic FM-CBAL74 against pediatric infectious diseases. Then, the lack of data regarding antagonistic effect against specific pathogens relevant for the pediatric age, and the current Coronavirus disease (COVID-19) pandemic determined by the global spreading of SARS-CoV-2 infection inspired the design of the two aims of the project.

The first aim of this project was to investigate the non-immune and immune mechanisms possibly regulated by FM-CBAL74 against *Rotavirus* (RV), the main agent responsible for acute gastroenteritis in the pediatric age.

The second aim was to investigate the possible protective action elicited by the postbiotic FM-CBAL74 against SARS-CoV-2, the agent of COVID-19.

3.0 Materials and Methods

Study Aim #1

To explore the potential protective action elicited by the postbiotic FM-CBAL74 against the main agent of RV infection we investigated several non-immune and immune mechanisms.

Rotavirus, a non-enveloped double-stranded RNA (dsRNA) virus of Reoviridae family, is the most common pathogen identified in children with acute gastroenteritis (AGE) worldwide [71]. It is the main cause of hospitalization for AGE and continues to be the leading cause of diarrhea-associated mortality among children younger than 5 years [72,73].

Acute gastroenteritis is responsible for an estimated 128 500 deaths and for more than 258 million episodes of diarrhea among children <5 years of age worldwide in 2016 [73].

Rotavirus is approximately 75 nm in diameter and has multilayered icosahedral protein capsid composed of an outer layer, an inner layer, and a core. The 18.5 kb genome is made up of 11 segmented dsRNA encoding six structural proteins (VP1-VP4, VP6, VP7) and six nonstructural proteins (NSP1-NSP6). *Rotavirus* infects the non-dividing mature endothelial cells at the tip of the villi that are present in the small intestinal lumen [74] leading to slight lesions, such as enterocyte vacuolization and loss or larger changes, such as villus blunting and crypt hyperplasia[75].

To limit RV diarrhea-associated mortality, in 2009 the World Health Organization (WHO) recommended the use of RV vaccine in all regions worldwide [76].

Although, a positive impact of RV vaccination has been demonstrated, the incidence and mortality rate of RV infection in high- and low-income locations remain to be different from each other.The reasons for the lower effectiveness of RV vaccination in low-income countries are at present not fully understood [77]. This scenario strongly suggests the use of anti-RV compounds to decrease the number of child deaths because of AGE-induced dehydration [78].

The postbiotic FM-CBAL74 could be a disrupting nutritional strategy against one of the most common killers for the pediatric age.

Human enterocytes

For all experiments, we used a well validated model of human enterocytes, the Caco-2 cell line (ATCC: American Type Culture Collection, Middlesex, UK; accession number: HTB-37). This experimental model is commonly used for research activities in this area. These cells conserve the typical features of small intestine human enterocytes [79] and they are commonly adopted for experiments exploring the effects of pathogens, drugs, probiotics, and nutrients on human intestine [80,81].

The Caco-2 cells were cultured in high glucose Dulbecco's modified Eagle medium (DMEM; Gibco, Berlin, Germany) 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 4 mM L- Glutamine (Sigma-Aldrich; St. Louis, Missouri, USA). Caco-2 cells were grown in an incubator at atemperature of 37 °C and CO_2, 5%. The culture medium was changed every 2 days. All experimentswere carried out in triplicate and three times.

Rotavirus strain

The simian *Rotavirus* strain SA11 was provided by ATCC (Accession number: VR-1565™). Virus stock was grown in MA104 cells (ATCC; accession number: CRL-2378.1™), which were maintained in Medium 199 (Lonza, Basel, Switzerland) without serum and supplemented with 20 µg/mL of trypsin from porcine pancreas type IX (Sigma-Aldrich, St. Louis, Missouri, USA) for 96h. After, the cells were lysed by freezing and thawing to achieve virus release. Extracted virus was titrated by focus forming assay (FFA) and expressed in focus-forming units (ffu) per cell, as previously described [82].

This strain is well characterized and is able to replicate to high titers in Caco-2 cells compared to other *Rotavirus* strains [83].

Study products

The fermented milk was prepared from skimmed milk fermented with *L.paracasei* CBA L74 (FM-CBAL74), as previously described. The control (non-fermented milk, NFM) consisted of skimmed milk powder with the same basal nutrients' composition of fermented milk powder (grams per 100g): proteins, 35; lipids, 1; carbohydrates, 54 (Table 2).

Rotavirus activation and infection protocol

Rotavirus strain SA11 activation was activated with 20 µg/mL trypsin from porcine for 1 hour at 37°C. The viral suspension was added to the apical side of cell monolayers. After 60 min, the cells were washed and incubated in serum-free medium for the indicated time periods after infection. We adopted two infection protocols: i) in the first infection protocol, FM-CBAL74 and NFM at the dose of 11.5 mg/ml were co-incubated with RV (25 ffu/cell), previously activated, in a sterile tube for 1h and then, used to stimulate the cells for 48 h; ii) in the second infection protocol, Caco-2 cells were pre-treated with 11.5 mg/ml of FM-CBAL74 and NFM for 48 hours at 37 °C prior the infection. The dose of 11.5 mg/mL has been established based on dose-response experiments and on previously results obtained by our group [34]. Then, the viral suspension (25 ffu/cell) was added to the Caco-2 monolayer for 1 hour. After inoculation, cells were washed twice to remove free viruses and maintained with serum-free medium after infection.

Quantification of Rotavirus infection

To quantify RV infection, undifferentiated Caco-2 cells were fixed with ice-cold methanol (Carlo Erba Reagents; MI, Italy) for 10 min, washed twice with phosphate-buffered saline (PBS) (Gibco, Berlin, Germany) and permeabilized for 10 min with 0,1 % Triton X-100 (A4975,0500; PanReac AppliChem) diluted in PBS. The cells were then washed with PBS and blocked with 1% bovine serum albumin (BSA; A6588,0050; PanReac AppliChem) in PBS-Tween 20 (A4974,0500; PanReac AppliChem) for 1 hour at room temperature. The samples were incubated with the specific primary antibody overnight at 4° C: anti-VP6 (1:100; ab181695; Abcam). After washing twice with PBS, the samples were incubated with the secondary antibody at room temperature for 1 hour: Donkey Anti-Mouse IgG Antibody, Alexa Fluor 594 (1:500; A-21203; Invitrogen, MA, USA). Nuclei were stained with 4'6-Diamidino-2-phenylindole dihydrochloride (DAPI) (1:1500; H3570; Thermofisher). Finally, cells were washed and mounted with antifading Mowiol (Sigma-Aldrich; St. Louis, Missouri, USA) and analyzed using an inverted fluorescence microscope.

Apoptosis assay

Apoptotic cells were identified by double staining with the Annexin V Apoptosis Detection Kit (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, after 48 hours of treatment, the cells were washed with PBS and incubated with 1x Annexin V binding buffer, then cells were stained with Annexin V-fluorescein isothiocyanate (FITC) for 10 min at room temperature in the dark. Before reading with a BD FACs Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), propidium iodide (PI) 5 µg/mL was added.

Transepithelial electrical resistance measurement

To evaluate the monolayer integrity by transepithelial electrical resistance (TEER), Caco-2 cells per well were seeded on polycarbonate 6-well Transwell® membranes (Corning, Life Science, Kennebunk, USA). After 15 days' post-confluence, the TEER of monolayer was measured every 24 hours for a total of 72 hours, after RV infection, using an epithelial Volt-Ohm Meter (Millicel-ERS-2, Millipore, Billerica, MA, USA). The measured resistance value was multiplied by the area of the filter to obtain an absolute value of TEER, expressed as Ω x cm^2.

The TEER values were measured as follows: (measured resistance value−blank value) × single cell layer surface area (cm^2).

Reactive oxygen species production

Intracellular reactive oxygen species (ROS) level, on differentiated Caco-2 cells, was detected by 2′, 7′-dichlorodihydrofluorescein (DCFH-DA), which is oxidized into fluorescent dichlorofluorescein (DCF) in the presence of ROS. Caco-2 cells were cultured in 24-well plates at a density of 7.5×10^4 cells/mL. After the stimulation, DCFH-DA (20 μM) was added for 20 min at 37°C in the dark and then the cells gently washed with PBS. Fluorescence of the cells was measured immediately on a microplate reader (SFM 25, Kontron Instruments; Japan) (Ex (λ) 485 nm; Em (λ) 535 nm). As a positive control, hydrogen peroxide (H_2O_2) (Sigma-Aldrich) was used at concentrations of 10 mM for 15, 30 and 60 min.

Quantitative real-time PCR

Total RNA was isolated from cells with TRizol reagent (Sigma-Aldrich) and quantified using a NanoDrop Spectrophotometer and purity was verified by A260:280 and A260:230 absorbance

ratios. The integrity of the RNA was checked using gel electrophoresis. Total RNA (1 μgr) was reverse transcribed in cDNA with a High-Capacity RNA-to-cDNA^{TM} Kit (Applied Biosystems; Vilnius, Lithuania) according to the manufacturer's instructions. Complementary DNA (cDNA) was stored at -80°C until use. Quantitative real-time PCR (qRT-PCR) analysis was performed using Taqman Gene Expression Master Mix (Applied Biosystems, Grand Island, NY, USA) to evaluate the effect of intestinal exposure to milk products and *Rotavirus* SA11 on the gene expression of TJ occludin (Hs00170162_m1 and Hs01551861_m1, respectively). The TaqMan probes for this gene were inventoried and tested by Applied Biosystems manufacturing facility (QC).

VP6 expression was evaluated using a SYBR green Master Mix (Applied Biosystems, Grand Island, NY, USA) with the following primers: VP6: 5′-GCACAGCCATTCGAACATCATGC-3′ (forward); 5′-TGCATCGGCGAGTACAGACTC-3′ (reverse).

Amplification conditions were initial steps at 50 °C for 2 min and 95 °C for 10 min, followed by 40cycles of 95 °C for 15 s and 60 °C for 1 min in a Light Cycler 7900HT (Applied Biosystems).

Data analysis was performed using the comparative threshold cycle (CT) method and expressed as $2^{-\Delta\Delta CT}$: ($\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{control}$).

Glyceraldeide-3-Phosphate Dehydrogenase (GAPDH) gene was used as the housekeeping gene (TaqMan probe: Hs02786624_g1; SYBR green 5′-AATCCCATCACCATCTTCCAG-3′ (forward); 5′-AATGAGCCCCAGCCTTC-3′ (reverse). Each sample was analyzed in triplicate.

Analysis of pro-inflammatory cytokines production

The concentrations of IL-8, IL-6 and TNF-α were analyzed in cell supernatants collected after treatment and stored at -80°C. The three cytokines production was measured by ELISA using commercially available kits (Abcam, Cambridge, USA) according to the manufacturer's instructions, and results expressed in pg/mL.

The detection limits of IL-8, IL-6 and TNF-α were 1.8pg/mL, 2 pg/mL and 30 pg/mL, respectively.

Western blot analysis

Western blotting analysis was carried on proteins lysed in cell lysis buffer [50 mMTris-Hcl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0,1% (w/v) SDS], supplemented with a protease and phosphatase inhibitor cocktail (Roche). The protein concentrations were determined by Bradford assay (Bio-Rad). The cell lysates (30 µg) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently blotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon^{R}-Transfer Membrane, Tullagreen, Carrigtwohill, Co). After 1 hour in blocking solution with 5% (w/v) non-fat dry milk (PanReac AppliChem) in PBS, containing 0.2% (v/v) Tween 20 (PBS-Tween), the PVDF membranes were incubated with the specific primary antibody overnight at 4 °C: anti-p-ERK1/2 (Thr202/Tyr204) (1:1000; ab32538; Abcam), anti-total ERK1/2 (1:1000; ab17942; Abcam), anti-p-JNK1+JNK2+JNK3 (T183+T183+T221) (1:1000; ab124956; Abcam), anti-total JNK1+JNK2+JNK3 (1:1000; ab179461; Abcam), anti-α-Tubulin (1:5000; T6074; Sigma-Aldrich), or anti-GAPDH (1:5000; G8795; Sigma-Aldrich). The membranes were then incubated with the required secondary antibodies for 1 hour at room temperature: Goat Anti-Rabbit IgG Antibody, HRP-conjugated (1:2000; ab205718; Abcam) or Goat Anti-MouseIgGAntibody, HRP-conjugated (1:5000; GtxMu-003-DHRPX; ImmunoReagents,), diluted in 5% (w/v) nonfat dry milk in PBS-Tween. The protein bands were visualized by chemiluminescence solution (Wester Antares; Cyanagen). The relative band intensity of each protein was normalized with those of the α-tubulin or GAPDH loading controls, using Image Lab software (Biorad, Hercules, CA, USA). Western blotting was performed in triplicate. Densitometry analyses shown were derived from three independent experiments.

Immunofluorescence and confocal microscopy

For actin cytoskeleton detection, undifferentiated Caco-2 cells were fixed with 4% paraformaldehyde (PFA) (Carlo Erba Reagents) for 30 min at room temperature. Autofluorescence due to free aldehyde groups from PFA treatment were blocked with 50 mM Ammonium Chloride (Sigma-Aldrich) in PBS for 10 min at room temperature. Cover slips were washed twice with PBS, then cells were permeabilized for 10 min with 0,1% Triton X-100 in PBS. All the samples were incubated in blocking solution containing 1% BSA in PBS-Tween 20 for 1 hour at room temperature. After that, the cells were orderly incubated with phalloidin-TRITC (1:50;P1951; Sigma-Aldrich) for 1 hour at room temperature. After thoroughly washing with PBS, the samples were mounted with antifading Mowiol. Glass slides were allowed to cure overnight, in the dark.

To investigate tight junctions (TJs) and adherent junctions' protein (AJ), Caco-2 cells were fixed, respectively, with ice-cold methanol and 4% PFA for 10 min or 30min at room temperature. Then, the cells were washed twice with PBS and permeabilized for 10 minwith 0,1% Triton X-100 in PBS. After washing, the cells were blocked using 1% BSA in PBS-Tween for 1 hour at room temperature and then incubated overnight at 4 °C with specific primary antibody: anti-occludin (1:100; ab31721; Abcam), anti-ZO-1 (1:200; ab96587; Abcam), and anti-E- cadherin (1:100; 610181; BD Biosciences). Coverslips were washed with PBS and incubated with the required secondary antibodies for 1 hour at room temperature: Donkey Anti-Rabbit IgG Antibody, Alexa Fluor 488 (1:200; A-21206; Invitrogen) or Goat Anti-Mouse IgG Antibody, Alexa Fluor 488 (1:400; A-11029; Invitrogen). Nuclei were stained with DAPI.

Finally, cells were mounted in Mowiol. Glass slides were allowed to cure overnight, in the dark.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). Fluorescence images presented are representative of cells imaged in at least three independent experiments.

Statistical analysis

All data were collected in a dedicated database and analyzed by a statistician using GraphPad Prism 7 (La Jolla, CA, USA). The Kolmogorov–Smirnov test was used to determine whether variables were normally distributed. Descriptive statistics were reported as means and standard deviations (SDs) for continuous variables. To evaluate the differences among continuous variables, data were analyzed using the one-way ANOVA test. The level of significance for all statistical tests was two-sided, $p<0.05$.

Study Aim #2

The second part of the PhD research project was focused on the investigation of the potential protective action elicited by the postbiotic FM-CBAL74 against SARS-CoV-2, the agent of Coronavirus disease (COVID-19).

Coronavirus disease is an ongoing pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [84]. SARS-CoV-2 is a single-stranded RNA (ssRNA) virus which infects epithelial cells of the respiratory tract, causing typical signs such as fever, dry cough, fatigue, and dyspnea. A small fraction of patients however progresses towards a severe form of pneumonia requiring the hospitalization or in some cases intensive care unit treatments [85].

Gastrointestinal tract is also a target organ for SARS-CoV-2 infection [86].
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 [35, 87,88.]

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 [89,90].

Viral infection leads to the release of massive amounts of inflammatory cytokines, defined as "cytokine storm" [91,92]. The cytokine storm attempts to eliminate the infecting virus, but, in the process, collateral damage to human tissue occurs as well [93].

The postbiotic FM-CBAL74 could be an alternative strategy for fighting SARS-CoV-2 infection.

Sars-CoV-2

Wild-type (WT) SARS-CoV-2 was isolated from the nasopharyngeal swab of a patient with laboratory-confirmed COVID-19, as previously described [94]. 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. Vero E6 cells were used for virus isolation from nasopharyngeal swabs. 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% CO_2 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 biosafety level-3 (BSL-3) authorized laboratory.

Cells infection protocol

Caco-2 cells were cultured in 6-well plates and were pretreated for 48 h with 11.5 mg/mL of FM-CBAL74 or NFM. This cellular model has been recently used to explore SARS-CoV-2 infection and potential therapeutic strategies against COVID-19 [95,96].

The dose of 11.5 mg/mL has been established based on dose–response experiments and previous data obtained by our group [34]. The cells were infected with 0.1 MOI of viral particles that belonged to the WT SARS-CoV-2 for 72 h. Subsequently, the cells were washed with PBS, and then harvested in TRizol or fixed in 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.

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

To determine the infectivity of SARS-CoV-2, Caco-2 cells were fixed with 4% PFA for 30 min at room temperature and processed for immunofluorescence staining to visualize viral antigen expression (Nucleocapsid protein, N). Autofluorescence due to free aldehyde groups from PFA treatment was blocked with 50 mM ammonium chloride 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 in PBS for 10 min. After washing, the cells were blocked for 1 h using 1% BSA in PBS-Tween and then incubated overnight at 4°C with specific primary antibody: anti-SARS-CoV-2 nucleoprotein (1:100; 3851; ProSci). Coverslips were washed with PBS and incubated with the required secondary antibodies for 1 hour at room temperature: Donkey Anti-Mouse IgG Antibody, Alexa Fluor 594 (1:500; A-21203; Invitrogen). Nuclei were stained with DAPI.
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 Liferiver 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 (×45cycles)]. The quantification cycle values of N were reported as means ± SD normalized to the internal control provided by the kit.

ACE2, TMPRSS2 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 (Hs00174179_m1 and Hs01122322_m1, respectively; Thermofisher).

Inflammatory cytokines expression (IL-6, IL-15, IL-1β, VEGFB, 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 2 . The cDNAs were amplified using a 7900 Real-Time PCR System (Applied Biosystems) at this cycling conditions: 50 °C for2 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-ΔCT. Beta-glucuronidase (GUSB) gene was used as the housekeeping gene:

5′-GAAAATATGTGGTTGGAGAGCTCATT-3′ (forward); 5′-CCGAGTGAAGATCCCCTTTTTA-3′ (reverse).

Expression data were normalized to non-infected cells (NI). Each sample was analyzed in triplicate.

Western blot analysis

Western blotting analysis was carried on proteins lysed in cell lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0,1% (w/v) SDS], supplemented with a protease and phosphatase inhibitor cocktail (Roche). The protein concentrations were determined by Bradford assay (Bio-Rad). The cell lysates (30 µg) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently blotted

onto polyvinylidene fluoride (PVDF) membranes (ImmobilonR-Transfer Membrane, Tullagreen, Carrigtwohill, Co). After 1 hour in blocking solution with 5% (w/v) non-fat dry milk (PanReac AppliChem) in PBS, containing 0.2% (v/v) Tween 20 (PBS-Tween), the PVDF membranes were incubated with the specific primary antibody overnight at 4 °C: anti-ACE2 (1:1000; ab15348; Abcam), or anti-β-actin (1:5000; ACTBD11B7; Santa Cruz, CA, USA). The membranes were then incubated with the required secondary antibodies for 1 hour at room temperature: Goat Anti-Rabbit IgG Antibody, HRP-conjugated (1:2000; ab205718; Abcam), or Goat Anti-Mouse IgG Antibody, HRP-conjugated (1:5000; GtxMu-003-DHRPX; ImmunoReagents,), diluted in 5% (w/v) nonfat dry milk in PBS-Tween. The protein bands were visualized by chemiluminescence solution (Wester Antares; Cyanagen). The relative band intensity of each protein was normalized with those of the α-tubulin or GAPDH loading controls, using Image Lab software (Biorad, Hercules, CA, USA). Western blotting was performed in triplicate. Densitometry analyses shown were derived from three independent experiments.

Statistical analysis

All the data were collected in a dedicated database and analyzed using the GraphPad Prism 7.0 software (La Jolla, CA, USA). 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, $p<0.05$.

4.0 Results

Study Aim #1

In preclinical and clinical studies, it has been demonstrated that a specific functional food, 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 [32,34].

In addition, the consumption of FM-CBAL74 has been associated with a positive modulation of gut microbiome structure and function in children and neonates [2,35].

In this PhD project, we investigated the potential protective action elicited by the postbiotic FM-CBAL74 against the main agent of RV infection on non-immune and immune mechanisms.

Cellular damage

Rotavirus infectivity is commonly demonstrated by the detection of viral capsid protein VP6 in human enterocytes [97], using immunofluorescence staining protein [82].

The infectivity of RV in Caco-2 cells was confirmed by the quantification of VP6 protein (Figure 4A-B), and mRNA levels (Figure 4C). The addition of FM-CBAL74 to RV for up to 6 hours before infection or pre-incubation with FM-CBAL74 for 48 hours before enterocytes RV infection were unable to limit the number of infected cells (Figure 4A-B).

The enterocyte is the main cell target for RV replication in the host and AGE is the principal clinical outcome of RV infection. A mechanism by which RV infection induces cell injury in human enterocytes is the alteration of the cytoskeleton, leading to a disorganization of F-actin [98].

Dynamic remodeling of the F-actin is regulated by ERK activation *via* phosphorylating some actin cross-linking/bundling proteins [99].

As shown in Figure 4D, non-infected cells showed a regular distribution of cytoskeleton actin filaments. In contrast, RV-infected enterocytes showed a marked alteration of cytoskeleton structure with a disorganization of F-actin filaments (Figure 4D). Pre-incubation with FM-CBAL74, but not with non-fermented cow's milk (NFM), protected the cells against RV-induced rearrangements of F-actin filaments (Figure 4D).

Rotavirus infection induces apoptosis in human intestinal Caco-2 cells [34,100]. An increase in necrotic cells (positive only for propidium, PI) and late apoptotic cells (positive for both PI and Annexin V) confirmed the pro-apoptotic effect induced by RV infection compared to non-infectedcells (Figure 4E-I). Pre-incubation with FM-CBAL74, but not with NFM, prevented these effects,down-regulating the percent of apoptotic cells *($p<0.05$)* (Figure 4E-I).

These results suggested that, while FM-CBAL74 is unable to prevent RV infection in human enterocytes, it can limit the subsequent cytoskeleton alterations and apoptosis.

MAP kinases pathway activation

Mitogen-activated protein kinase (MAPK) cascades are key signaling pathways that regulate a wide variety of cellular processes, including proliferation, differentiation, apoptosis, and stress responses [101]. A typical MAPK cascade consists of at least three sequentially acting serine/threonine kinases, aMAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and finally, the MAP kinase (MAPK) itself, with each phosphorylating, and hence activating, the next kinase in the cascade [102].

There are three well-characterized subfamilies of MAPKs in mammals: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 kinases [103].

Some studies have showed that ERK signaling contribute to cell survival, whereas JNK and p38 signaling contributes to cell death. Recently, has been shown that ERK activation not always correlated with cell survival; but also, this MAP kinase would show an interaction with cell death, including apoptosis, autophagy, and senescence [104].

To further characterize the intracellular mechanisms involved in the protective action of FM-CBAL74, we investigated the MAP kinase pathway activation. As shown in Figure 5A-B, RV infection resulted in an increase ratio of phosphorylation of extracellular signal-regulated kinases (ERK) and c-Jun-N-terminal kinase (JNK), demonstrating an activation of this pathways. The pre-treatment with FM-CBAL74 prevented the increase of phosphorylation ratio of ERK and JNK induced by RV compared to NFM *($p<0.001$)*.

These results provided evidence on ERK/JNK pathway involvement in the protective action of FM-CBAL74 against RV infection.

Intestinal Permeability

To investigate the protective action of FM-CBAL74 against gut barrier alteration induced by RV infection, we evaluated the transepithelial electric resistance (TEER), the expression of TJ proteins, occludin and zonula occludens 1 (ZO-1), and of AJ protein, E-cadherin, in Caco-2 cell monolayer.

As shown in Figure 6A, RV infection determined a significant decrease of transepithelial resistance (TEER) in human enterocytes ($p<0.05$). This event was associated with an alteration of TJ proteins, as demonstrated by redistribution of occludin and ZO-1 (Figure 6B and Figure 6C), and the reduction of their expression ($p<0.05$) (Figure 6B and Figure 6C). The pre-treatment with FM-CBAL74, but not with NFM, prevented RV-induced TEER decrease *($p<0.05$)* (Figure 6A).

Moreover, the pre-treatment with FM-CBAL74 compared to NFM, protected Caco-2 cells from occludin and ZO-1 redistribution and enhanced their expression *($p<0.05$)* (Figure 6B and Figure 6C, suggesting a protective effect against gut barrier dysfunction.

Furthermore, we found that E-cadherin expression appeared significantly down-regulated in RV-infected cells compared to non-infected cells ($p<0.001$) (Figure 6D). Again, pre-treatment with FM-CBAL74 significantly prevented the reduction of E-cadherin expression caused by RV infection compared to NFM *($p<0.05$)* (Figure 6D).

Inflammatory response

Reactive oxygen species, also referred to as reactive oxygen intermediates (ROI), which includes oxygen, superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide, are chemical molecules produced by cellular metabolism [105]. Low and moderate amounts of ROS have beneficial effects on several physiological processes including killing of invading pathogens, wound healing, and tissue repair processes [106]. An overproduction of ROS can become lethal and destroy the structure of biological macromolecules such as DNA and proteins, leading to various diseases including cancer, respiratory, cardiovascular, neurodegenerative, digestive diseases, and oxidative stress [107]. Furthermore, the increased levels of inflammatory mediators could be followed by higher expression of cytokines including interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) [108.] These cytokines are well known pro-inflammatory cytokines involved in local and systemic inflammatory reactions [109].

To further study the protective effect of FM-CBAL74 on intestinal epithelial oxidative stress and pro- inflammatory cytokines response induced by RV infection, we analyzed ROS and IL-6, IL-8 and TNF-α cytokines production.

As shown in Figure 7A, RV significantly increased ROS production. The pre-treatment with FM-CBAL74, but not with NFM, inhibited the RV-induced ROS increase *($p<0.05$)* (Figure 7A). IL-6, IL-8 and TNF-α production were significantly higher in the RV-infected cells than those in non-infected cells (Figure 7B-D). These effects were blunted by FM-CBAL74, demonstrating a protective effect of this postbiotic *($p<0.05$)* (Figure 7B-D).

Altogether these data suggested that FM-CBAL74 was able to inhibit ROS production and IL-8, IL-6 and TNF-α cytokines release induced by RV infection in human enterocytes.

Study Aim #2

This second part of the PhD research project was focused on the investigation of the potential protective action elicited by the postbiotic FM-CBAL74 against SARS-CoV-2, the agent of COVID-19. To assess the potential protective effect of FM-CBAL74 against SARS-CoV-2 infection, we investigated the N viral protein by immunofluorescence staining and RT-PCR in a well validated model of human enterocytes, the Caco-2 cell line.

Our study showed that the pretreatment with FM-CBAL74, but not with NFM, significantly reduced the amount of N mRNA and viral protein in Caco-2 cells infected with SARS-CoV-2 (Figure 8A-B). This result was probably due to a modulation of the SARS-CoV-2 receptor ACE2, the molecular mediator that facilitate 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 (Figure 8C-D). No effects have been observed on TMPRSS2 expression, suggesting that the preventive action of FM-CBAL74 not involved the modulation of TMPRSS2 (Figure 8C).

SARS-CoV-2 infection is, also, associated with high levels of pro-inflammatory cytokines. To investigate 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 [110], and we observed that this particular fermented food was able to significantly reduce the expression of IL-6, VEGFB, IL-15, and IL-1β (Figure 8E).

These results showed that modulating the crucial aspects of the infection, the fermented food FM-CBAL74 exerts a preventive action against SARS-CoV-2 (Figure 9).

This evidence could pave the way to innovative nutritional strategy to mitigate the COVID-19.

5.0 Discussion

Emerging evidence suggest the potential of the postbiotic approach for the prevention of pediatric infectious diseases [31-35,111-114.]

Unfortunately, discrepancies in clinical results evaluating different postbiotic products, and the paucity of preclinical data on the mechanisms of action elicited by these compounds against specific pathogens are still blunting the wide use of this approach.

This PhD research project aimed to explore the anti-RV and anti-SARS-CoV-2 effect of a specific postbiotic deriving from the fermentation of cow's milk with the probiotic FM- CBAL74 with a demonstrated clinical efficacy against pediatric AGE [32-33].

We found that this postbiotic positively modulate a range of immune and non-immune defense mechanisms against RV and SARS-CoV-2 infection in human enterocytes.

Regarding to the effects on RV, despite this postbiotic was unable to significantly reduce the number of infected cells, a protective action against gut barrier damage, characterized by cytoskeleton alterations and apoptosis, was observed. The effect involved the modulation of a pivotal regulator of stress-induced cell damage, the ERK/JNK kinase pathway [115].

ERK and JNK are members of MAP kinases, which are ubiquitous serine/threonine kinases that are involved in transduction of externally derived signals regulating many biological processes [116]. Many viruses, including influenza A, herpes simplex virus 1, hepatitis C, RV, employ the ERK/JNK pathway to facilitate different stages of their life cycle for host infection [117-119].

Rotavirus activated this pathway through the interaction of its outer capsid proteins, such as VP4-VP8 andVP4-VP5 domains and VP7, and with non-structural protein NSP1 [120].

MAP kinases activation and apoptosis induction in human enterocytes play also a role in the breakdown of epithelial barrier, through an altered distribution of occludin and ZO-1, as also

demonstrated by a reduction of TEER value [80,81,121].

The gut barrier integrity represents the first line of defense against pathogens targeting epithelial cells [122] and it is of pivotal importance in protecting children against gastrointestinal infections [123]. As reported by our study and by others, RV infection elicited an alteration of the gut barrier integrity, through structural and functional changes in TJ and AJ proteins in human enterocytes, as demonstrated by a decrease of TEER [34,123-125]. We found that FM-CBAL74 had beneficial effects on gut barrier integrity and on the increased enterocyte monolayer permeability in RV- infected cells, preventing RV-induced decrease in TEER and up-regulating occluding, ZO-1 and E-cadherin expression. These effects also could be due to a down-regulation of RV-induced MAP kinases pathway activity.

According to previous evidence [126,127], our data demonstrated that RV infection elicited an increase of ROS production in human enterocytes. ROS are involved in MAP kinase pathway activation [128], leading to pro-inflammatory cytokines release, including IL-8, IL-6 and TNF-α, [121,129-132].

We demonstrated that FM-CBAL74 was able to prevent RV-induced ROS, IL-8, IL-6 and TNF-α production, suggesting a potent inhibition of the inflammatory storm which in turn is responsible for the severity of signs and symptoms commonly observed in children infected by this microorganism [72]. We have investigated the protective anti-RV action of FM CBAL74 in an *in vitro* experimental tool. We have demonstrated that this postbiotic could exert a positive modulation of gut microbiome in the pediatric age associated with an increased production of secretory IgA (sIgA) and butyrate in the intestinal lumen [2,32,33-35].

Considering the relevant role exerted by these molecules in protecting against infections and in modulating gut barrier integrity and inflammation, it is possible to hypothesized that this postbiotic could modulate an even higher range of defense mechanisms against RV [113].

Other studies on the efficacy and mechanisms of action of FM-CBAL74 using more complex

systems, such as human biopsies and/or organoids exposed to different gastrointestinal pathogens, could be advocated to further explore the potential of such approach.

It has been hypothesized that fermented foods could be a potential strategy to also limit SARS-CoV-2 infection, but the mechanisms are still largely undefined [133].

In this PhD research project, 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-based postbiotic 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 [89,134]. Blocking of the viral receptor-binding domains represents a key step in antiviral approaches [135]. We found that FM-CBAL74 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 [135,136]. The GI tract contributes to the genesis of the "cytokine storm" [133].

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 [137]. The modulation of these cytokines is considered a relevant target to improve COVID-19 outcomes [138]. In our study, we observed that FM-CBAL74 was able to reduce the levels of IL-1β, IL6, VEGFB, 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 [139]. Accordingly, clinical trials are providing promising results on

the effects of IL-6 inhibitors in COVID-19 patients [140,141]. The research on active compounds able to counteract SARS-CoV-2 infection is very active [142].

Recently has been provided evidence on phosphate foods additives, including inorganic polyphosphates (polyPs) that at low dose are able to inhibit SARS-CoV-2 infection, replication, and cytokine storm [94]. The results of the present study provide additional evidence on the potential of nutritional strategies against COVID-19.

The limitations of this PhD research project, were that we did not provided evidence on which specific FM-CBAL74 components, deriving from the fermentation of cow's milk with the probiotic, 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. It has been demonstrated that peptides deriving from fermentation of cow's milk proteins could act as modulators of non-immune and immune gastrointestinal defense mechanisms [143-146].

Another limitation is related to the fact that we explored the protective effects of FM-CBAL74 against only the main agent of pediatric AGE, *Rotavirus*. Subsequent studies are necessary to explore the protective action elicited by FM-CBAL74 against other agents responsible for pediatric AGE. Furthermore, we did not provided evidence on other potential molecular mechanisms involved in SARS-CoV-2 infection.

Future studies are advocated to better define the protective action elicited by this postbiotic against human infectious diseases, and future clinical trials are advocated to explore the clinical impact of this nutritional strategy against pediatric AGE and COVID-19.

6.0 Conclusions

In conclusion, we provided evidence on the protective action elicited by FM-CBAL74 against RV and SARS-CoV-2 infection. Our findings could act in parallel with other beneficial actions on the structure and function of the gut microbiome and on innate and adaptive immunity, that have been shown in previous studies [2,32-35].

Altogether, this evidence could pave the way for innovative nutritional strategies against RV one of the most common pediatric killers, responsible for more than 200,000 deaths per year worldwide [73]; as well as to mitigate COVID-19 that is responsible for 5.6 million deaths worldwide up to now [147].

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9.0 Tables and Figures

Table 1. Ingredients utilized for cow milk fermentation

Ingredients	% w/v
Skimmed milk (powder)	10.5
Water	87.5
Dextrose	2

Table 2. Composition of the study dietary products

Value for 100 gr of product	Fermented milk with *L.paracasei* CBA L74	Non-fermented milk
Proteins, g	24.0	35.0
Carbohydrates, g	66.4	54.0
Fats, g	0.6	1.0
pH	6.2	6.2
L. paracasei CBA L74, CFU[1]	5.9×10^9	-

[1]Killed Bacteria

Figure 1. The plant located in the *"Kraft Heinz Innovation center"*

Figure 2. Figurative scheme of the plant

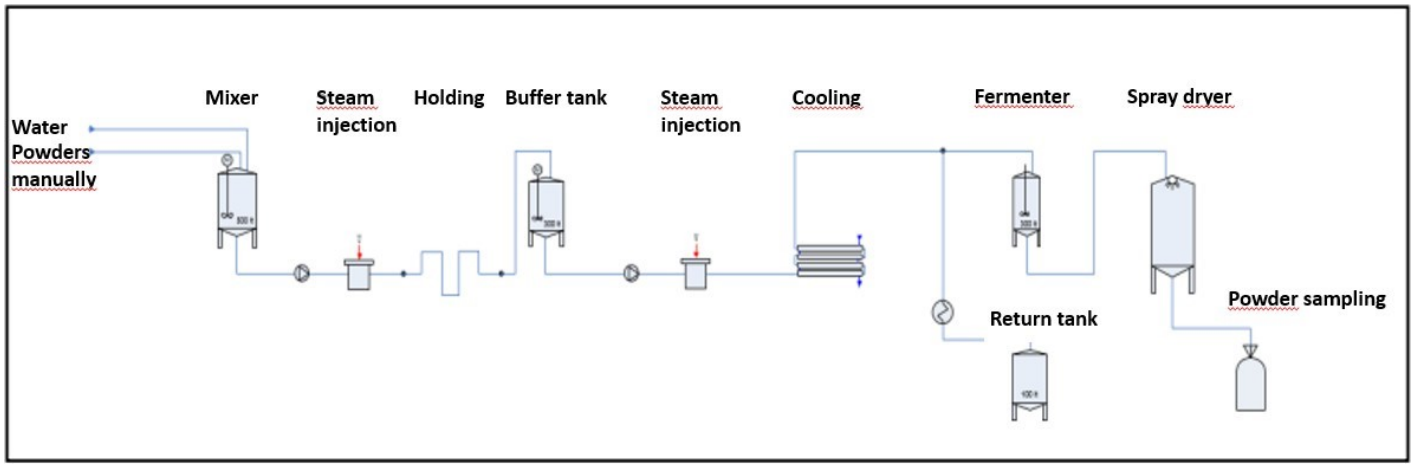


Figure 3. Milk fermentation flowchart

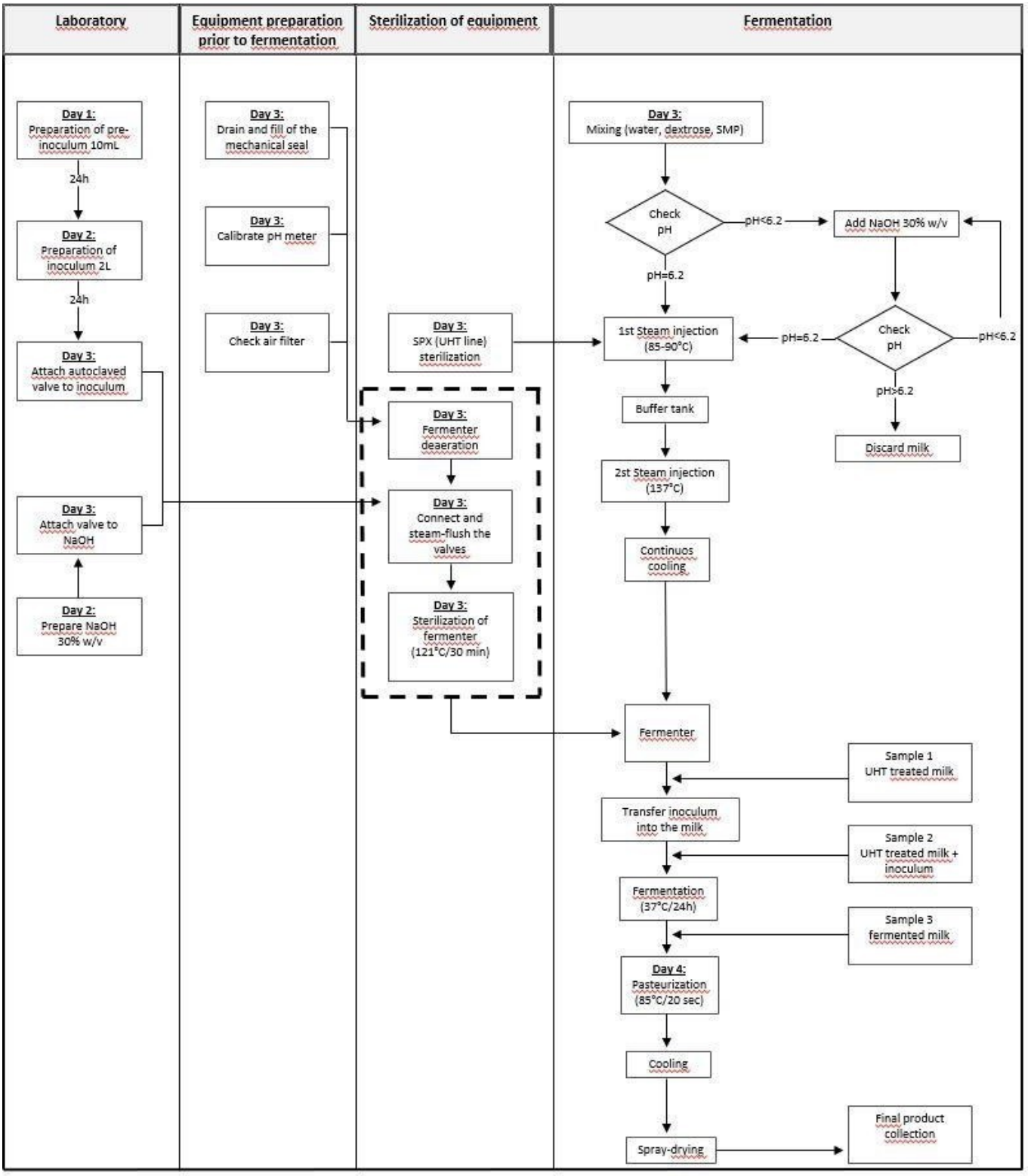
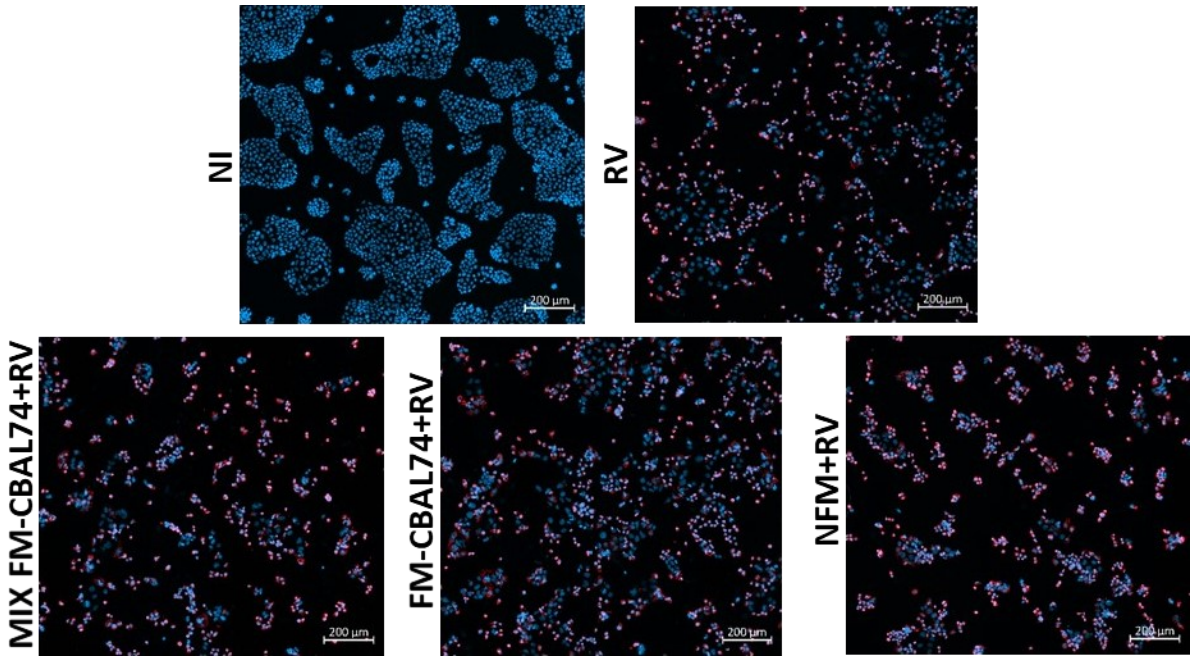


Figure 4. Effects of FM-CBAL74 on cellular damage in *Rotavirus*- infected human enterocytes

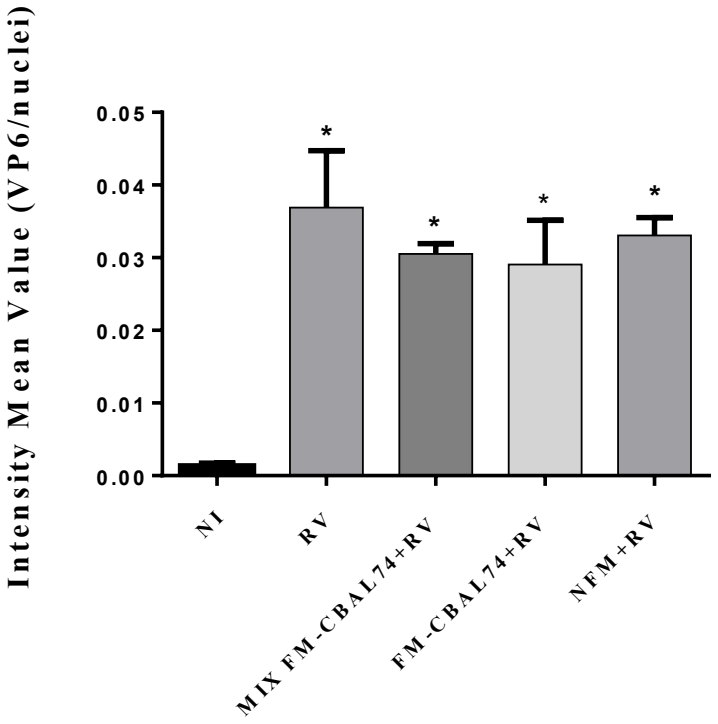
A)



(A) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. Cells were fixed and processed for immunofluorescence. VP6 protein was visualized using VP-6 Alexa Fluor-594 (red) and nuclei were stained with DAPI (blue). Cells were observed through confocal. The addition of FM-CBAL74 to RV for up to 6 hours before infection (MIX FM-CBA74+RV) or pre-incubation with FM-CBAL74 (FM-CBAL74+RV) resulted in no significant modulation of RV infectivity. Scale bar, 200 µm.

NI, non-infected cells; RV, Rotavirus; Mix FM-CBAL74+RV, fermented cow milk with FM-CBAL74 and RV; FM-CBAL74+RV, pre-treatment with FM-CBAL74 and infection with RV; NFM+RV, pre-treatment with NFM and infection with RV.

B)



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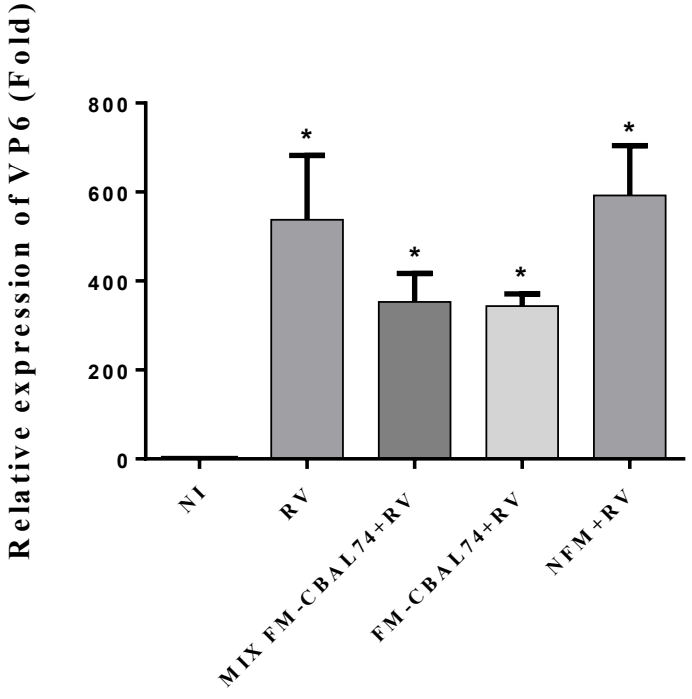
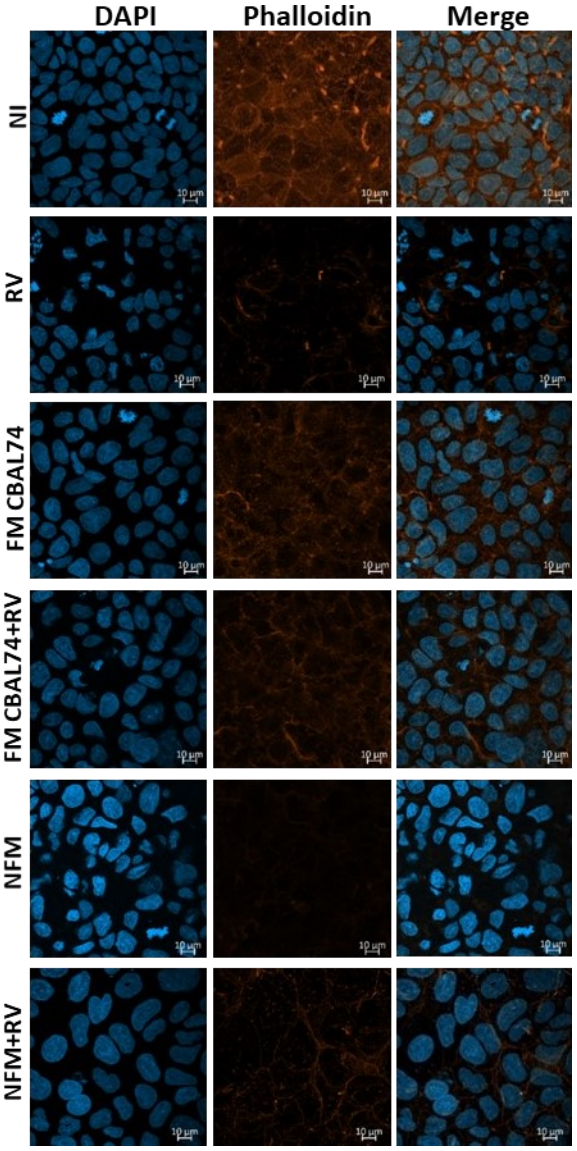


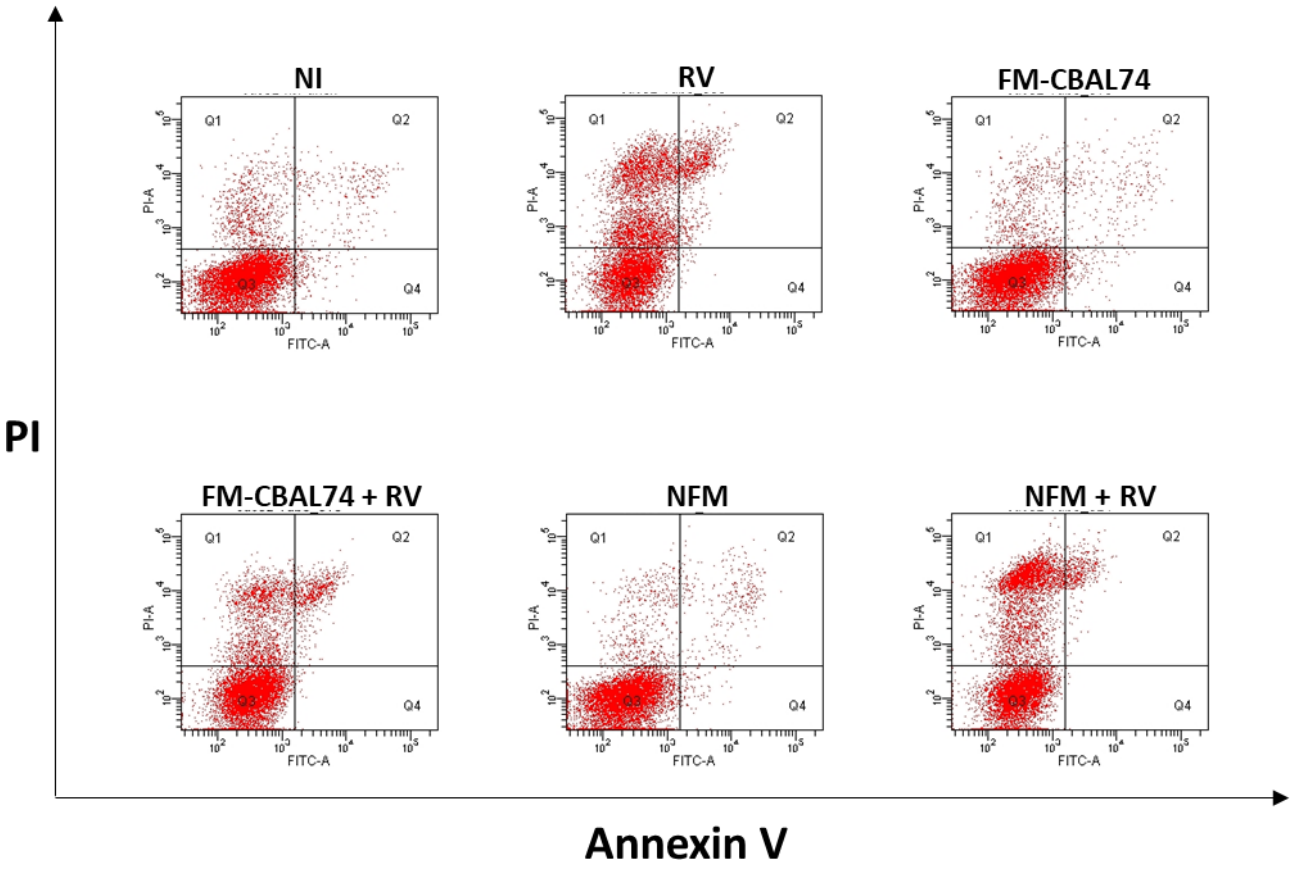
(B) Intensity mean value of VP6 are shown. **(C)** The quantification by VP6 by RT-PCR confirmed the results obtained in IF staining. Data represent the means with SD of 3 independent experiments, each performed in triplicate. Data were analyzed using the one-way ANOVA test. *p<0.05 *vs* NI; °p<0.05 *vs* NFM+RV. *NI, non-infected cells; RV, Rotavirus; Mix FM-CBAL74+RV, fermented cow milk with FM-CBAL74 and RV; FM-CBAL74+RV, pre- treatment with FM-CBAL74 and infection with RV; NFM+RV, pre-treatment with NFM and infection with RV.*

D)



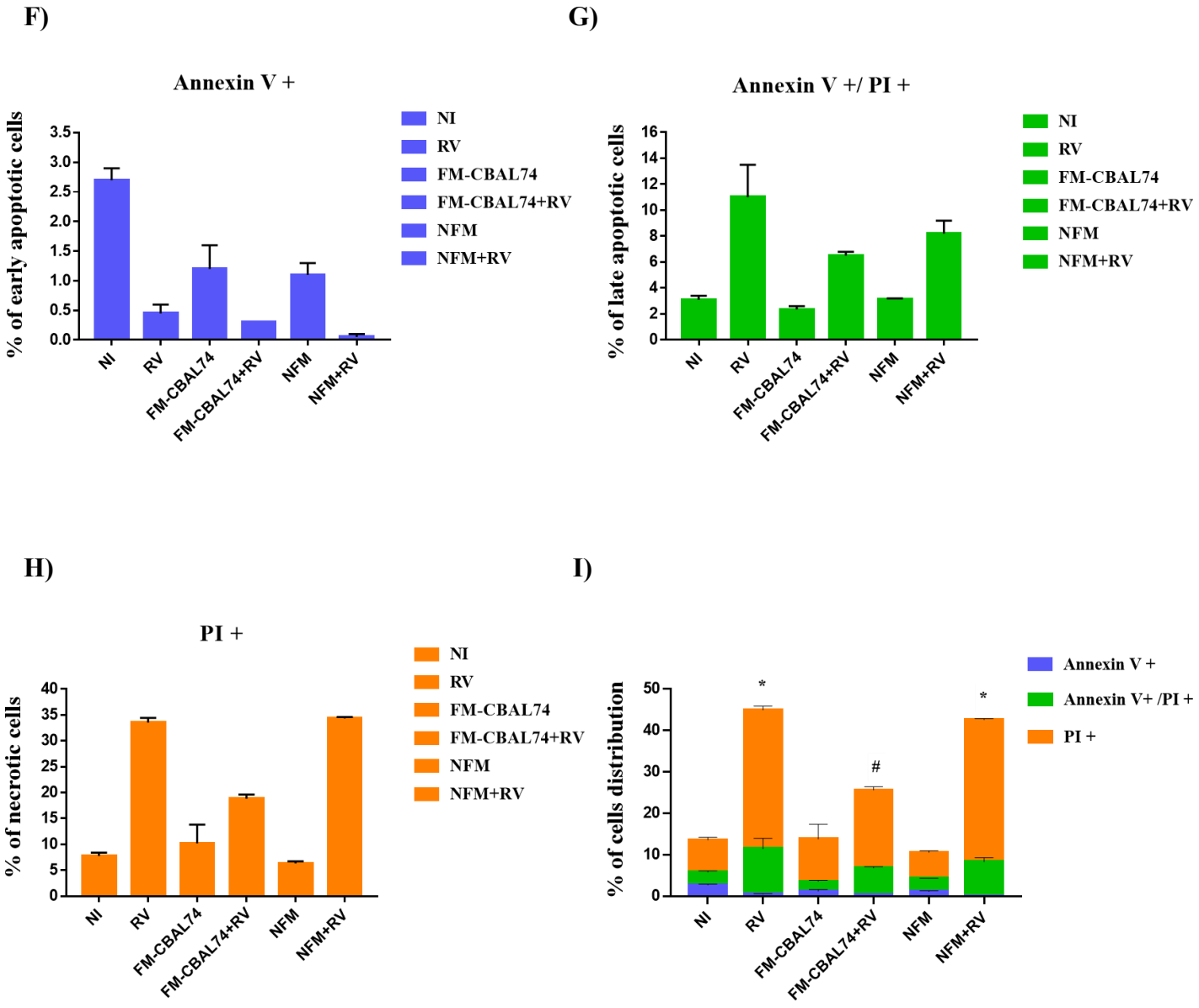
(D) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. Cells were fixed and processed for immunofluorescence. Actin was visualized using phalloidin- TRITC (red) and nuclei were stained with DAPI (blue). *Rotavirus*-infected enterocytes showed a marked alteration of cytoskeleton structure with a disorganization of F-actin filaments. NI showed a regular distribution of cytoskeleton actin filaments. Pre-incubation with FM-CBAL74 (FM-CBAL74+RV), but not with NFM (NFM+RV), protected the cells against RV-induced F-actin filaments rearrangements. Scale bar, 10 µm. *NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L. paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74 and infection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.*

E)



(E) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. Apoptotic cellrate was assessed by Annexin V assay using flow cytometry. An increase in necrotic cells (positive only for propidium, PI) and late apoptotic cells (positive for both PI and Annexin V) confirmed the pro-apoptotic effect induced by RV infection compared to non- infected cells. Pre-incubation with FM-CBAL74 (FM-CBAL74+RV), but not with NFM (NFM+RV), prevented these effects.

NI, non-infected cells; RV, *Rotavirus*; FM-CBAL74, fermented milk *L.paracasei* CBAL74; *FM-CBAL74+RV, pre-treatment with FM-CBAL74 and infection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.*

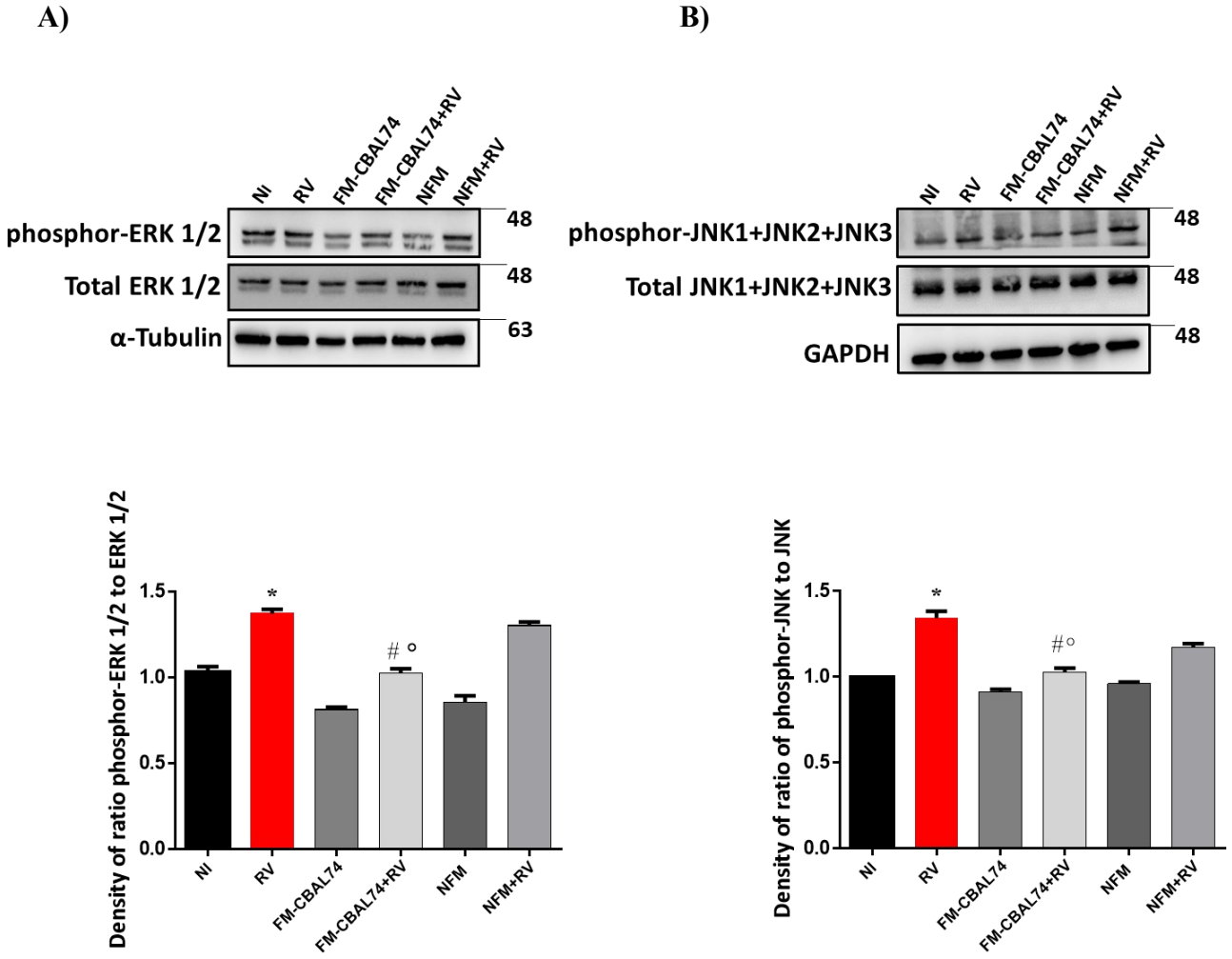


(F) The histograms show the percentage of cell distribution in early apoptosis (positive for Annexin V staining), **(G)** late apoptosis (double positive for both Annexin V and PI staining), **(H)** and necrotic cells (single positive for PI staining). **(I)** Summary graph of cell distribution in apoptosis analysis. Data represent the means with SD of 3 independent experiments, each performed in triplicate.

Data were analyzed using the one-way ANOVA test. *$p<0.05$ *vs* NI; $^{\#}p<0.05$ *vs* RV; °$p<0.05$ *vs* NFM+RV.

NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L.paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74 and infection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.

Figure 5. Effects of FM-CBAL74 on MAP kinases pathway activation in *Rotavirus*-infected human enterocytes

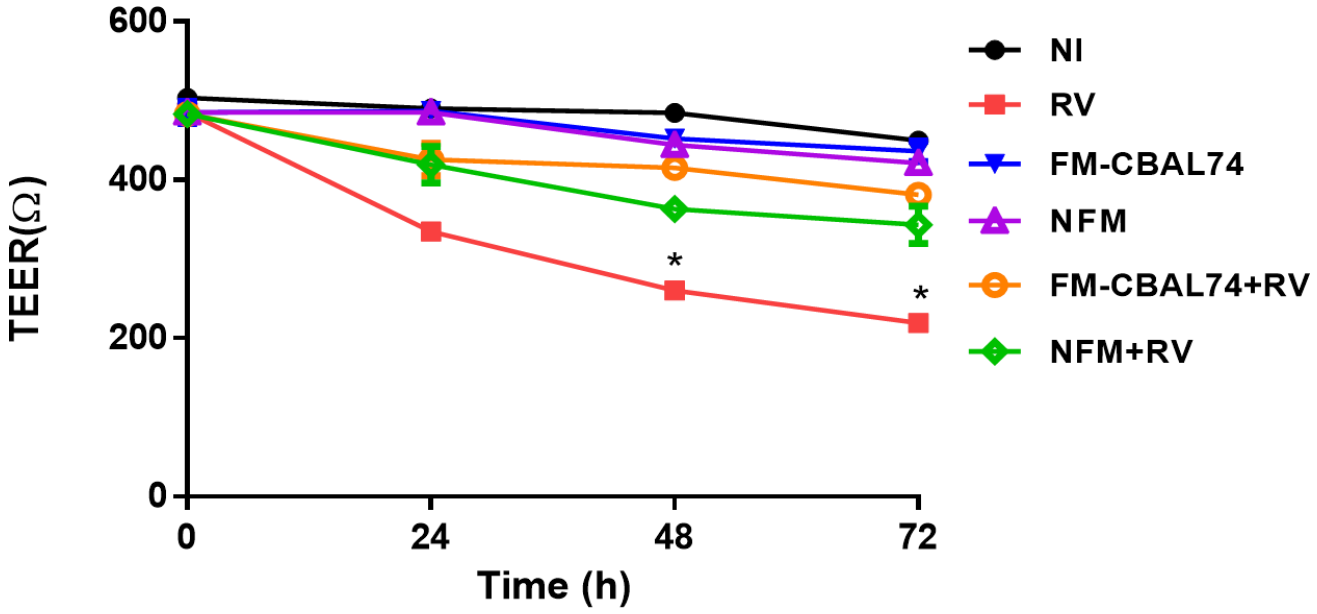


(A-B) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. Western blot assay of phosphor-ERK/total ERK **(A)** and phosphor-JNK/total JNK **(B)** was performed on protein extracts from Caco-2 cells. *Rotavirus* infection induced MAP kinases ERK, and JNK expression significantly increased compared with non-infected cells (NI). Pre-incubation with FM-CBAL74 (FM-CBAL74+RV), but not with NFM (NFM+RV), down-regulated this pro-inflammatory pathway in Caco-2 cells. The amounts of these proteins and of α-tubulin, and GAPDH, were measured by Western blot. The histogram below shows optical density of the proteins, obtained with Image Lab software. Relative quantification of proteins was normalized versus α-tubulin and GAPDH proteins and was calculated using the ratio between phosphorylated and total proteins. The figure showed representative image of three experiments qualitatively similar. Data represent the means with SD of 3 independent experiments, each performed in triplicate. Data were analyzed using the one-way ANOVA test. *$p<0.05$ *vs* NI; #$p<0.05$ *vs* RV; °$p<0.05$ *vs* NFM+RV.

NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L.paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74 and infection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.

Figure 6. Effects of FM-CBAL74 on gut permeability in *Rotavirus*-infected human enterocytes

A)

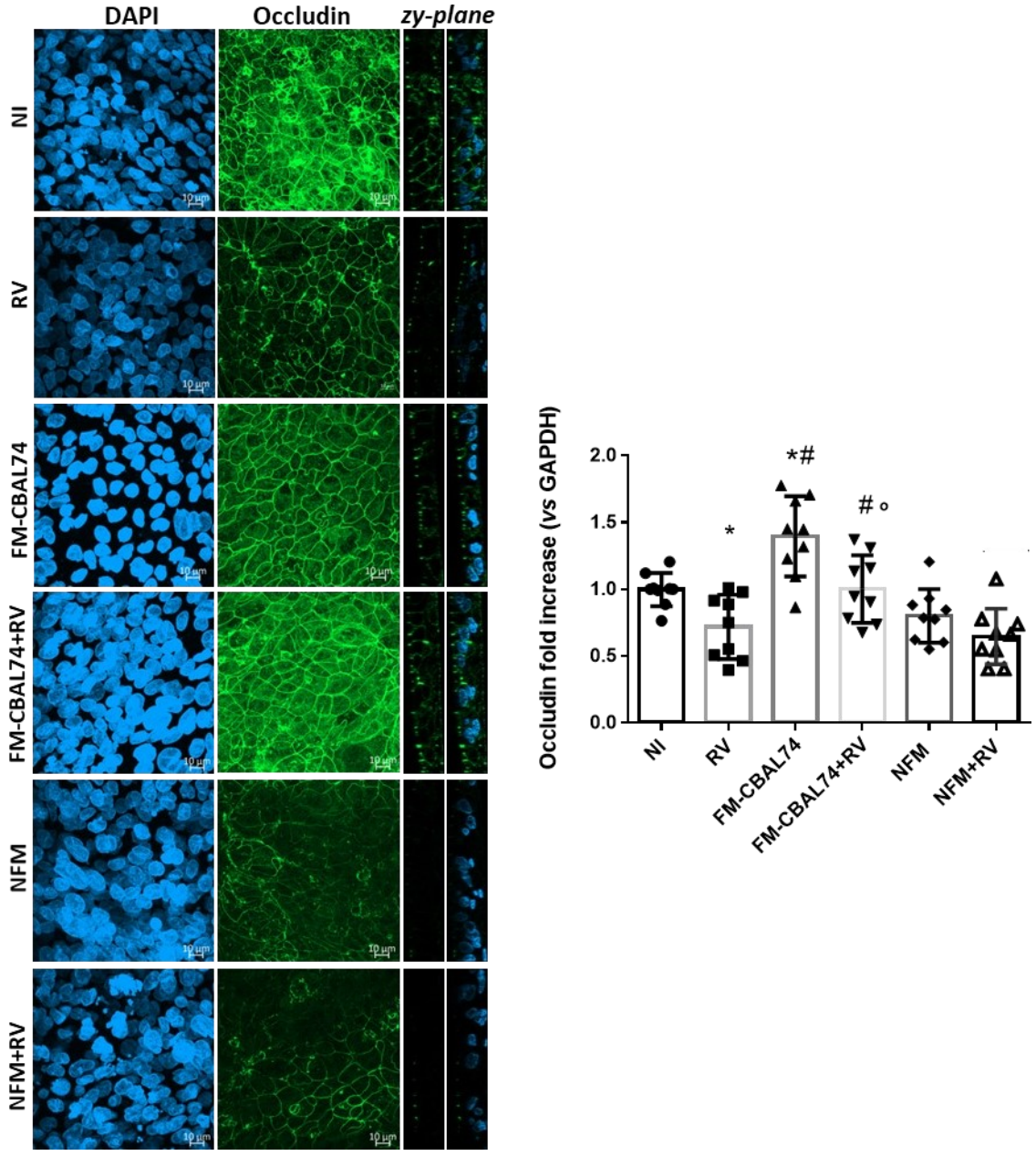


(A) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. *Rotavirus* infection affect intestinal epithelial permeability, as demonstrated by TEER measurement up to 72 hours of incubation. Pre- incubation with FM-CBAL74 (FM-CBAL74+RV) significantly inhibited this effect. Data represent the means with SD of 3independent experiments, each performed in triplicate.

Data were analyzed using the one-way ANOVA test. *p<0.05 *vs* NI; #p<0.05 *vs* RV; °p<0.05 *vs* NFM+RV.

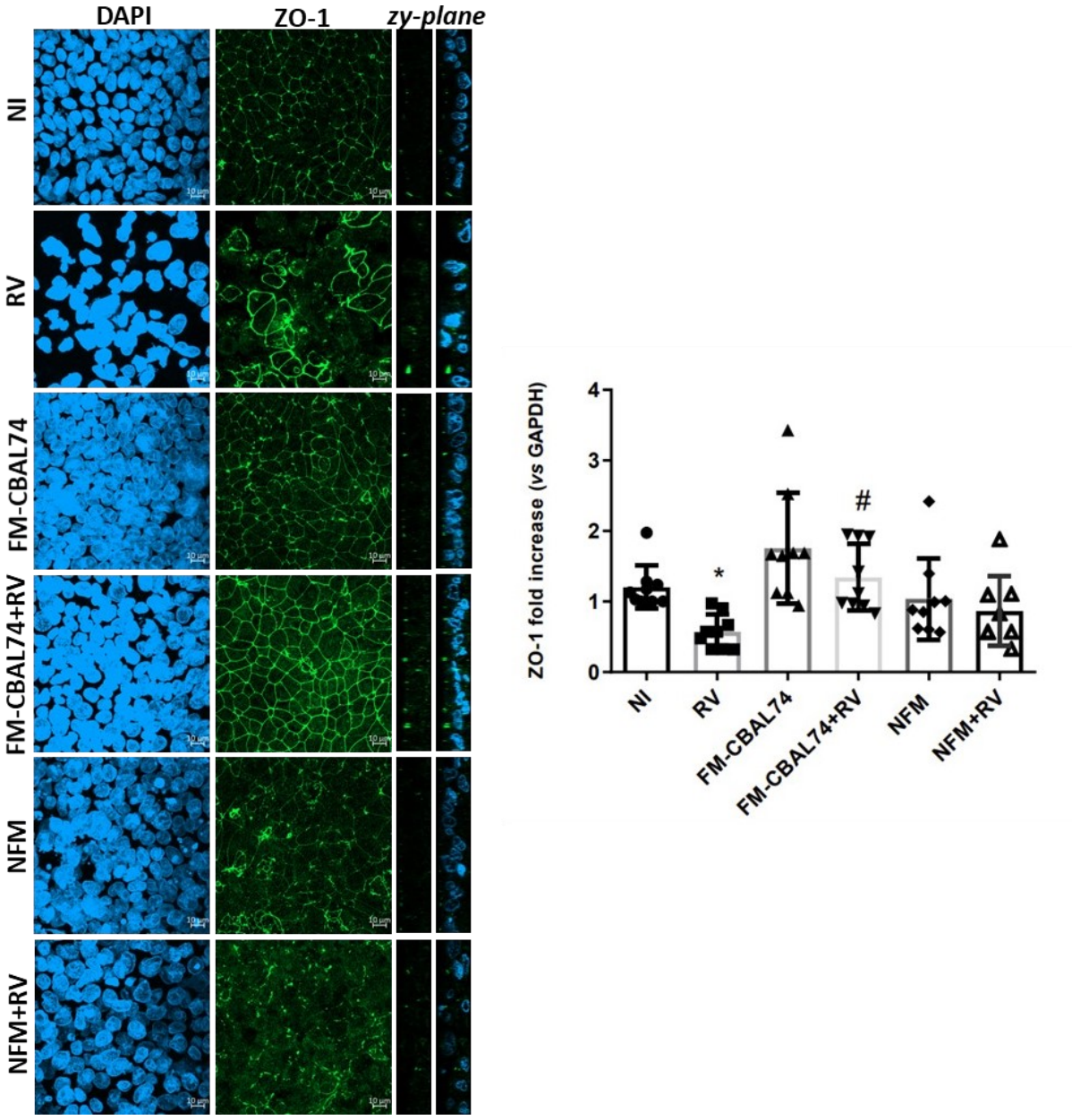
NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L. paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74and infection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.

B)



(B) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. Cells were processed for mRNA and immunofluorescence analysis. Occludin was visualized using anti-rabbit Alexa 488 (green) and nuclei were stained with DAPI (blue). Cells were observed through confocal microscope in the *zy-plane*. *Rotavirus* infection elicited a significant reduction of TJ occludin in Caco-2 cells compared to non-infected cells. Pre-treatment of RV-infected cells with FM-CBAL74 (FM-CBAL74+RV) significantly prevented occludin mRNA **(Right image)** and protein **(Left image)** reduction in Caco-2 cells monolayer. NFM was unable to modulate the expression of occludin in RV-infected cells. Scale bar, 10 µm. *p<0.05 *vs* NI; #p<0.05 *vs* RV; °p<0.05 *vs* NFM+RV. *NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L.paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74 and infection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.*

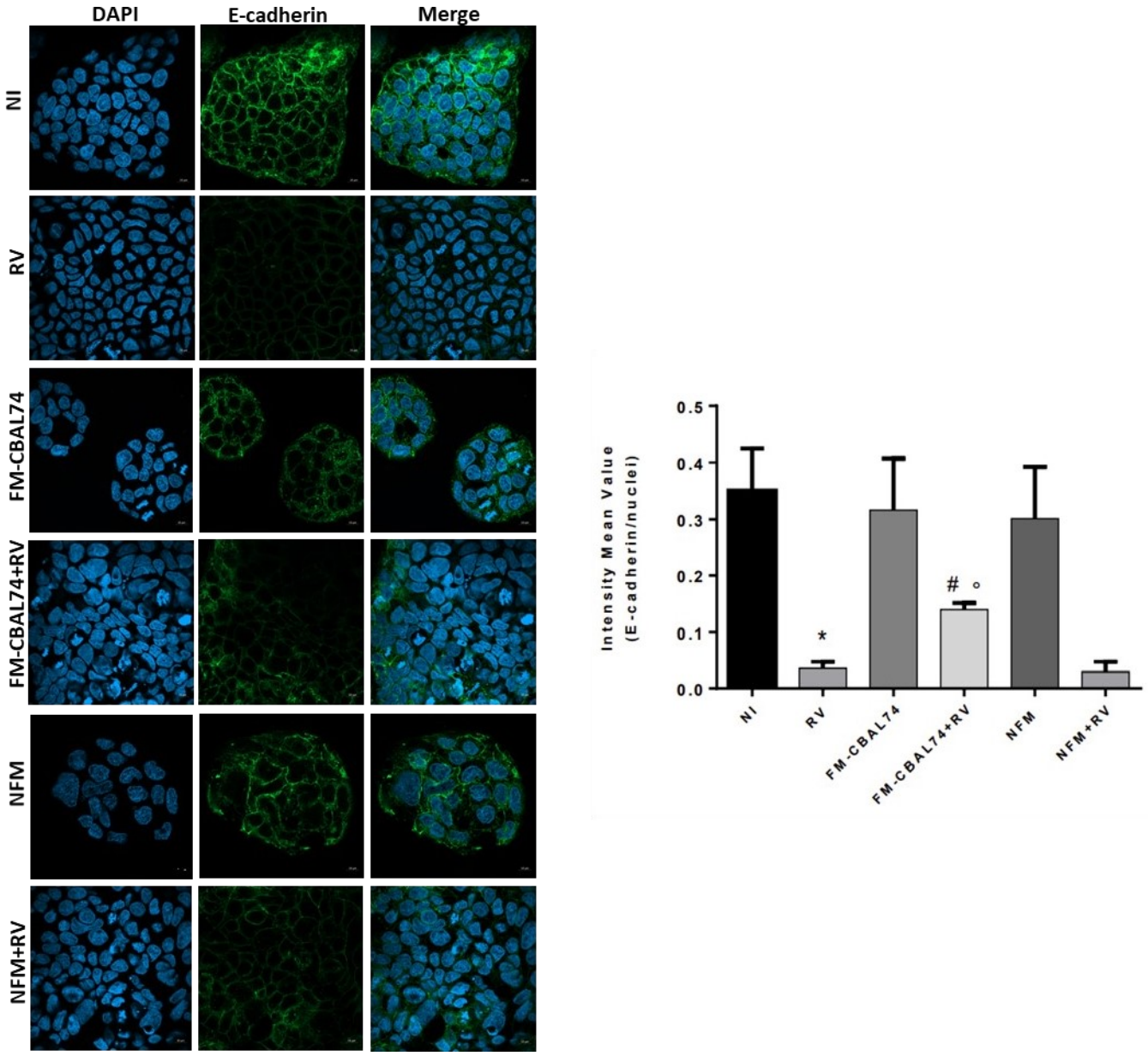
C)



(C) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. Cells were fixed and processed for immunofluorescence. ZO-1 was visualized using anti-rabbit Alexa 488 (green) and nuclei were stained with DAPI (blue). Cells were observed through confocal microscope in the *zy-plane*. *Rotavirus* infection elicited a significant reduction of ZO-1 in Caco-2 cells compared to non-infected cells. Pre-treatment of RV-infected cells with FM-CBAL74 significantly prevented ZO-1 mRNA **(Right image)** and protein **(Left image)** reduction in Caco-2 cells monolayer. NFM was unable to modulate the expression of ZO-1 in RV- infected cells. Scale bar, 10 µm. *p<0.05 *vs* NI; #p<0.05 *vs* RV; °p<0.05 *vs* NFM+RV.

NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L. paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74 andinfection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.

D) **E)**

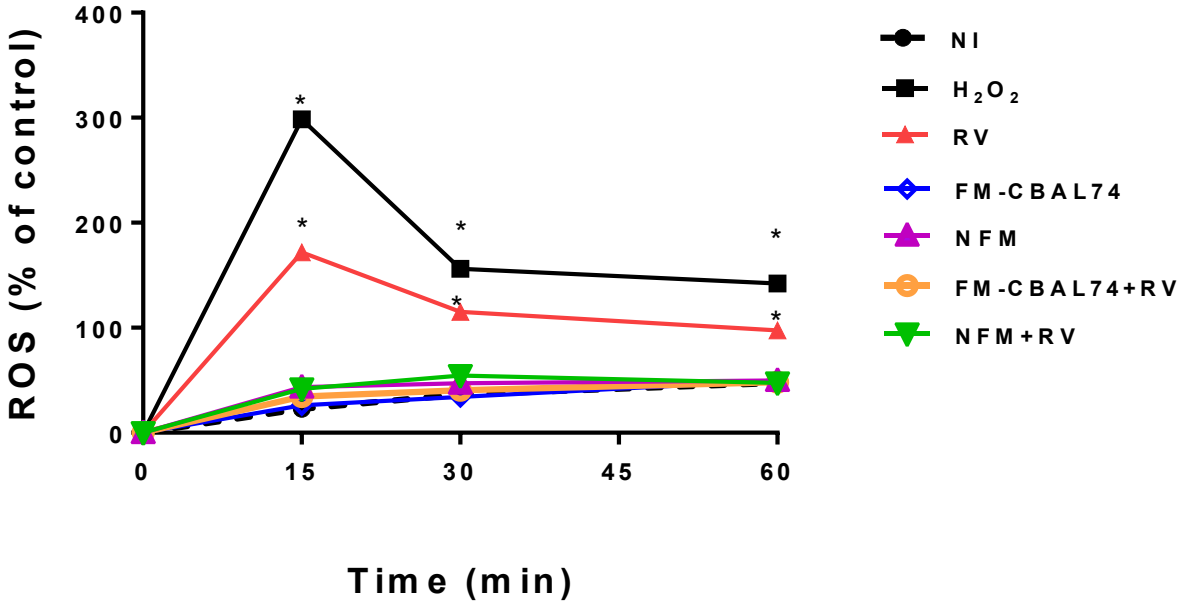


(D) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. Cells were fixed and processed for immunofluorescence. E-cadherin was visualized using anti-mouse Alexa 488 (green) and nuclei were stained with DAPI (blue). *Rotavirus* infection reduced E-cadherin expression compared to non-infected cells. Pre-incubation of RV-infected cells with FM-CBAL74 (FM-CBAL74+RV) significantly prevented the reduction of E-cadherin expression in Caco-2 cells monolayer. NFM was unable to modulate the expression of E-cadherin in RV-infected cells. **(E)** Intensity mean value of E-cadherin are shown. Scale bar, 10 µm. *p<0.05 *vs* NI; #p<0.05 *vs* RV; °p<0.05 *vs* NFM+RV.

NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L.paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74 and infection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.

Figure 7. Effects of FM-CBAL74 on ROS and pro-inflammatory cytokines production in *Rotavirus*-infected human enterocytes

A)



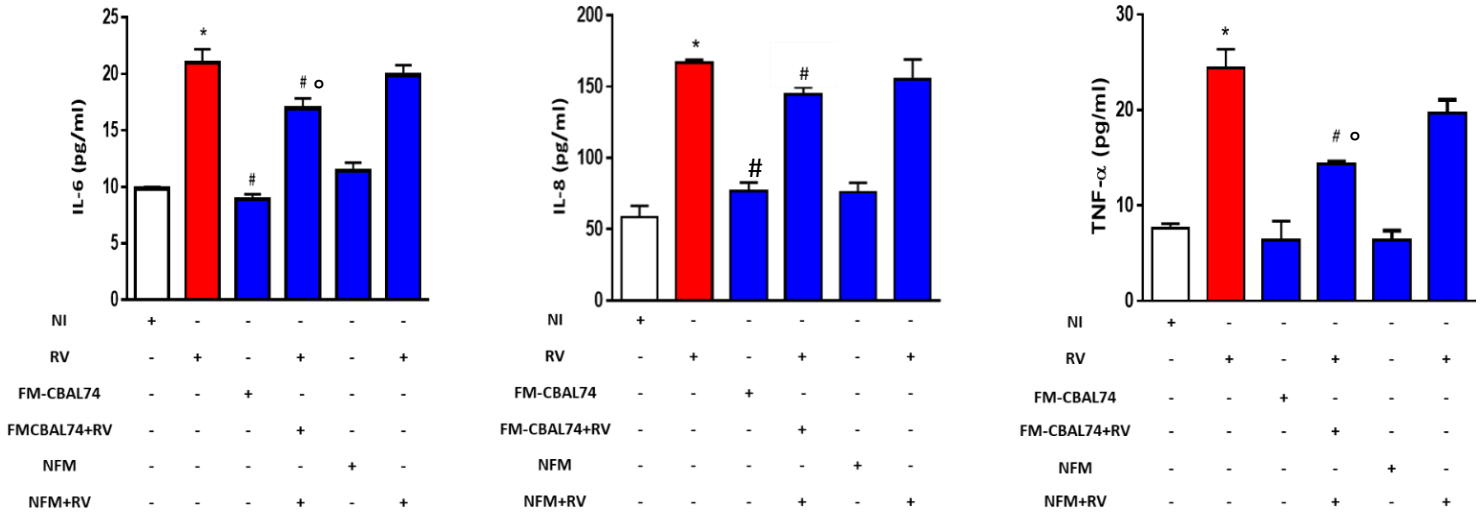
(A) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. *Rotavirus* induced a significant increase in ROS production in a time-dependent manner. Pre-incubation with FM-CBAL74 (FM-CBAL74+RV), but not with NFM (NFM+RV), significantly inhibited the RV-induced increase in ROS. H_2O_2 was used as a positive control.

Data represent the means with SD of 3 independent experiments, each performed in triplicate.

Data were analyzed using the one-way ANOVA test. *p<0.05 *vs* NI; #p<0.05 *vs* RV; °p<0.05 *vs* NFM+RV.

NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L. paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74 andinfection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.

B) **C)** **D)**



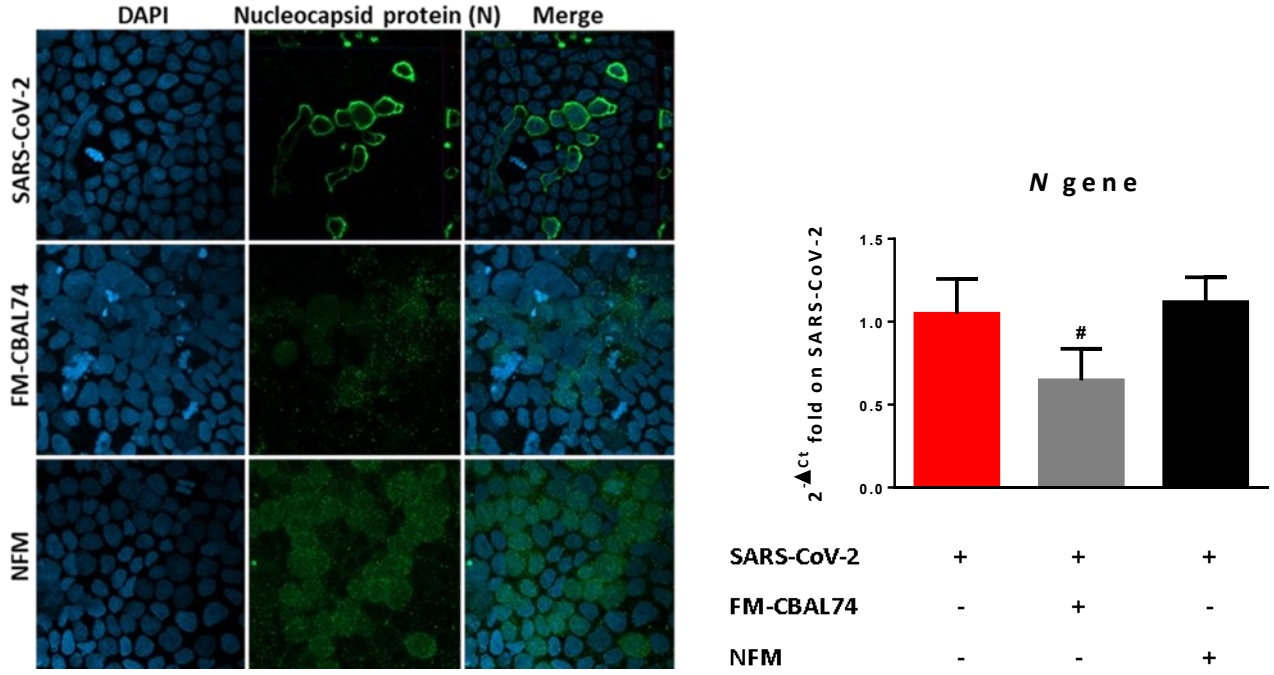
(B-D) Caco-2 cells were infected with RV at 25 ffu/cell and pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48h. The supernatants were collected for ELISA assays. *Rotavirus* elicited a significant increase in IL-6 **(B)**, IL-8 **(C)** and TNF-α **(D)** production. Pre-incubation with FM-CBA L74(FM-CBAL74+RV), but not with NFM (NFM+RV), significantly inhibited the RV-induced increase in IL-6, IL-8 and TNF-α production in Caco-2 cells. Data represent the means with SD of 3 independent experiments, each performed in triplicate. Data were analyzed using the one-way ANOVA test.

*p<0.05 *vs* NI; #p<0.05 *vs* RV; °p<0.05 *vs* NFM+RV.

NI, non-infected cells; RV, Rotavirus; FM-CBAL74, fermented milk L.paracasei CBAL74; FM-CBAL74+RV, pre-treatment with FM-CBAL74 and infection with RV; NFM, not fermented milk, NFM+RV, pre-treatment with NFM and infection with RV.

Figure 8. Effects of FM-CBAL74 on infectivity, ACE2, TMPRSS2 and pro-inflammatory cytokines expression in SARS- CoV-2 infected human enterocytes

A) **B)**

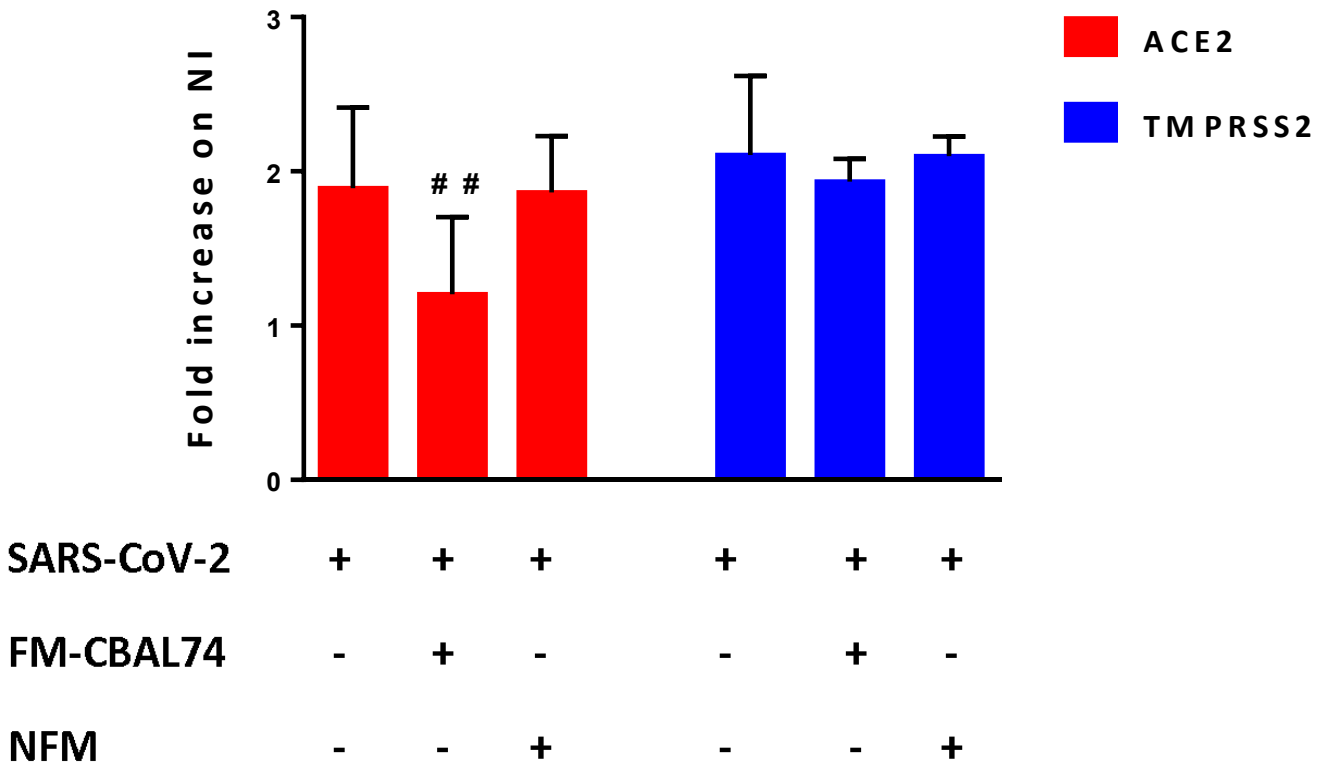


(A) Caco-2 cells were infected with 0.1 MOI of WT SARS-CoV-2 pretreated with 11.5 mg/mL of FM-CBAL74 or NFM for 48 h. Cells were fixed and processed for immunofluorescence. N protein was visualized using anti-mouse Alexa Fluor 594 (green) and nuclei were stained with DAPI (blue). Cells were observed through confocal microscopy. SARS-CoV-2 infection in Caco-2 cells was confirmed by the quantification of N protein by immunofluorescence staining. The pretreatment with FM-CBAL74 resulted in the reduction of SARS-CoV-2 infected cells. Scale bar, 20 µm.

(B) Quantification of N gene of viral RNA by RT-PCR analysis. The pretreatment with FM-CBAL74 inhibited N gene transcription in SARS-CoV-2 infected cells. Data were analyzed using one-way ANOVA test. #p = 0.011 *vs* SARS-CoV-2.

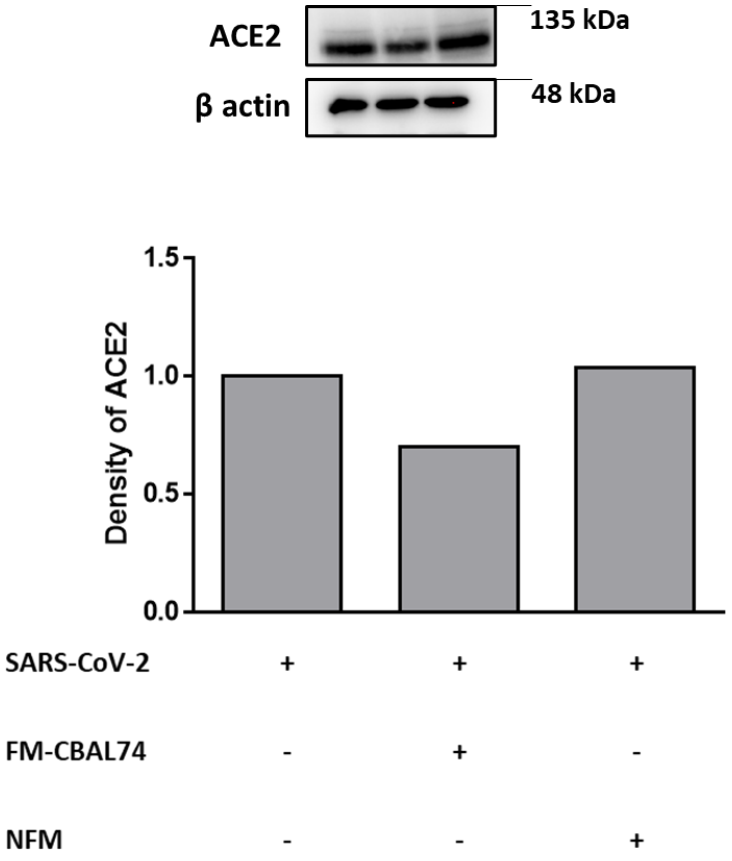
SARS-CoV-2, cells infected with WT SARS-CoV-2; FM-CBAL74, fermented milk L. paracasei CBA L74; NFM, not fermented milk.

C)



(C) Pre-incubation with 11.5 mg/mL of FM-CBAL74, but not with NFM, significantly down-regulated ACE2 expression in Caco-2 cells exposed toSARS-CoV-2. No modulation was observed for TMPRSS2 expression. Data represent the means with SD of 3 independent experiments, each performed in triplicate. Data were analyzed using one-way ANOVA test. ##p = 0.006 *vs* SARS-CoV-2.

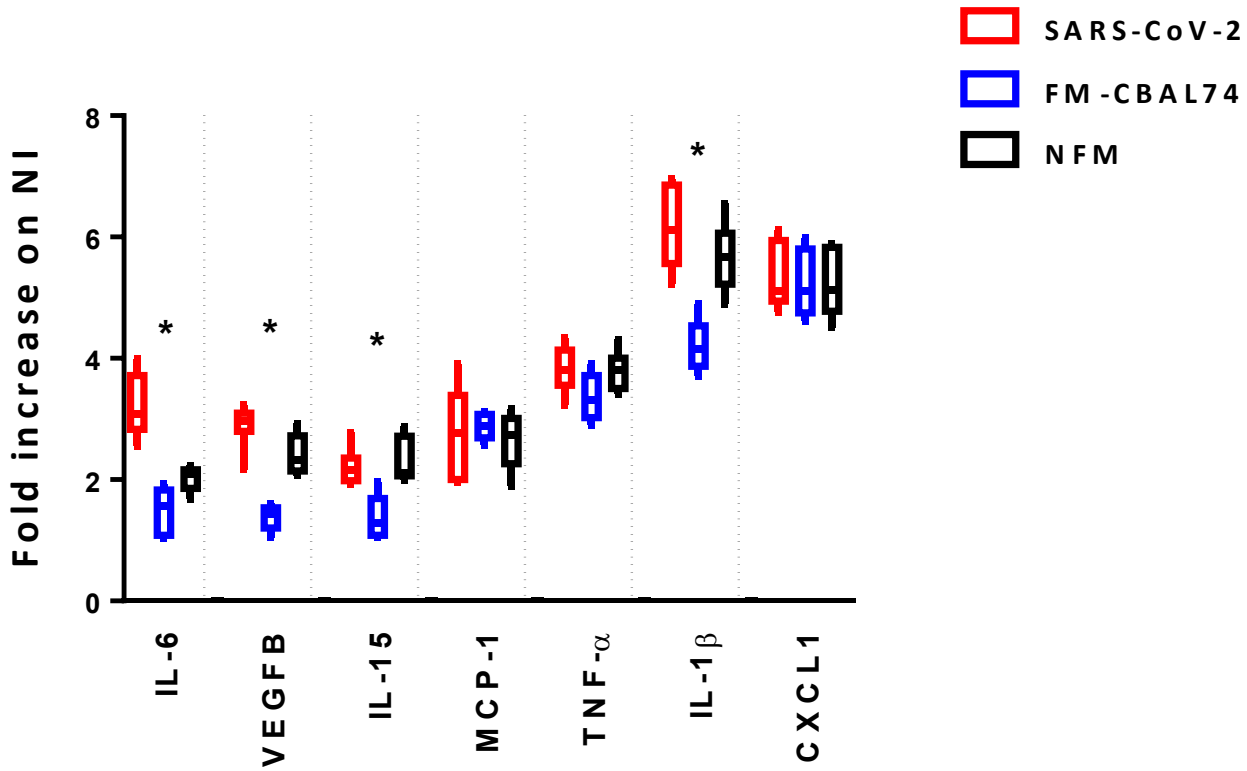
D)



(D) For ACE2 protein analysis, representative image of three experiments qualitatively similar was reported. Western blot assay of ACE2 was performed on protein extracts from SARS-CoV-2 infected Caco-2 cells. The amounts of ACE2 and β-actin were measured by Western blot. The histogram below shows optical density of the protein, obtained with Image Lab software. Pre-incubation with FM-CBAL74, but not with NFM, significantly reduced ACE2 protein level in Caco-2 cells exposed to SARS-CoV-2. Relative quantification of proteins was normalized versus β-actin protein and was calculated using SARS-CoV-2 as calibrator.

SARS-CoV-2, cells infected with WT SARS-CoV-2; FM-CBAL74, fermented milk L.paracasei CBA L74; NFM, not fermented milk.

E)



(E) Pre-incubation with 11.5 mg/mL of FM-CBAL74, but not with NFM, significantly reduced the IL-6, VEGFB, IL-15 and IL-1β expression in Caco-2 cells exposed to SARS-CoV-2. Data represent the means with SD of 3 independent experiments, each performed in triplicate.Data were analyzed using one-way ANOVA test. *$p < 0.001$ *vs* SARS-CoV-2.

SARS-CoV-2, cells infected with WT SARS-CoV-2; FM-CBAL74, fermented milk L.paracasei CBAL74; NFM, not fermented milk.

Figure 9. Fermented food FM-CBAL74 exerts a preventive action in SARS- CoV-2 infected human enterocytes

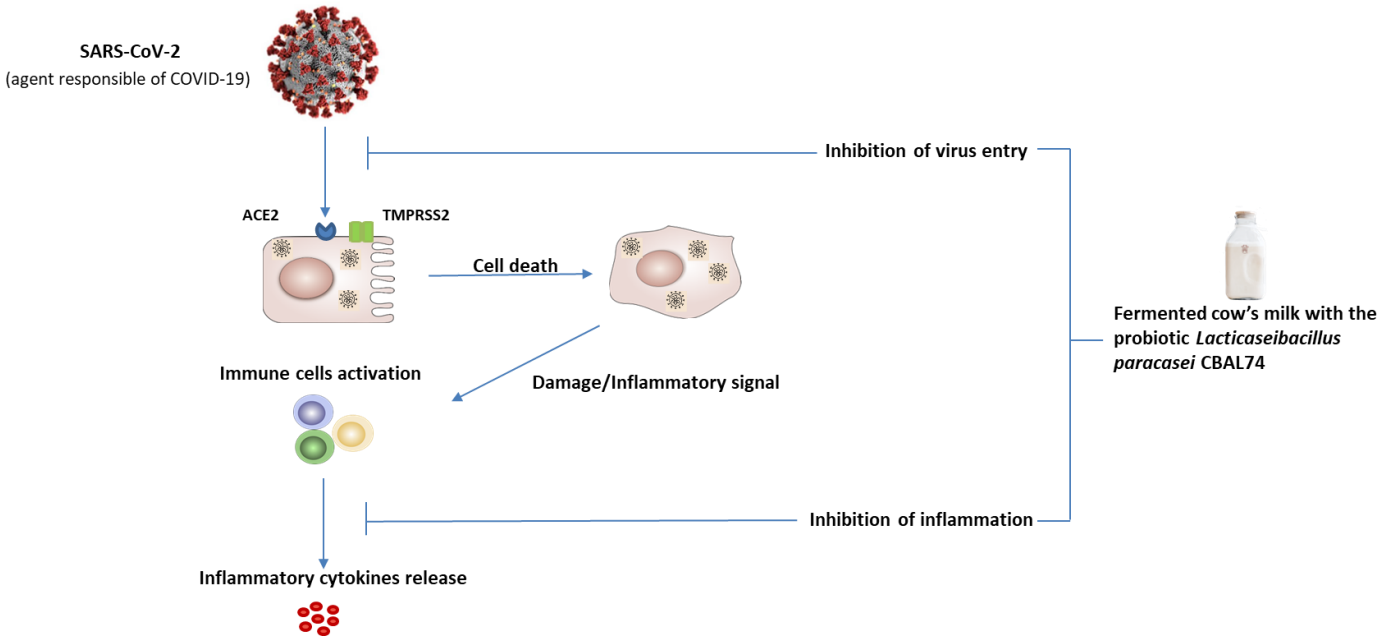


Table 3. Sequences of the primers used for the analysis of pro-inflammatory cytokines

Gene	Primer	Primer sequence
IL-6	Forward	CTCGACGGCATCTCAGCC
	Reverse	GCCTCTTTGCTGCTTTCACAC
IL-15	Forward	CAGTTGCAAAGTAACAGCAATGAA
	Reverse	GCATCTCCGGACTCAAGTGAA
IL-1β	Forward	CTTTGAAGCTGATGGCCCTAA
	Reverse	CGCCATCCAGAGGGCAG
VEGFB	Forward	AGAAGGAGGAGGGCAGAATCA
	Reverse	GATGGCAGTAGCTGCGCTG
TNF-α	Forward	CTTCTGCCTGCTGCACTTTG
	Reverse	TGATTAGAGAGAGGTCCCTGGG
MCP1	Forward	CTATAGAAGAATCACCAGCAGCAGCAAGT
	Reverse	TCTCCTTGGCCACAATGGTC
CXCL1	Forward	GCGCCCAAACCGAAGTCATA
	Reverse	ATGGGGGATGCAGGATTGAG

IL-6, interleukin-6; IL-15, interleukin-15; IL-1β, interleukin-1 beta; VEGFB, Vascular endothelial growth factor B; TNF-α, Tumor necrosis factor-alpha; MCP1, Monocyte chemoattractant protein-1; CXCL1, C-X-C Motif chemokine ligand 1.

10.0 Publications in the last 3 years

Adv Exp Med Biol - Advances in Microbiology, Infectious Diseases and Public Health (2019) 1125: 57–68
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Targeting Food Allergy with Probiotics

Lorella Paparo, Rita Nocerino, Carmen Di Scala, Giusy Della Gatta, Margherita Di Costanzo, Aniello Buono, Cristina Bruno, and Roberto Berni Canani

Abstract

The dramatic increase in food allergy prevalence and severity globally is demanding effective strategies. Food allergy derives from a defect in immune tolerance mechanisms. Immune tolerance is modulated by gut microbiota composition and function, and gut microbiota dysbiosis has been associated with the development of food allergy. Selected probiotic strains could act on immune tolerance mechanisms. The mechanisms are multiple and still not completely defined. Increasing evidence is providing useful information on the choice of optimal bacterial species/strains, dosage, and timing for intervention. The increased knowledge on the crucial role played by gut microbiota-derived metabolites, such as butyrate, is also opening the way to a post-biotic approach in the stimulation of immune tolerance.

Keywords

Butyrate · Cow's milk allergy · Gut microbiota · Immune tolerance · Post-biotics · Probiotics

Authors Lorella Paparo, Rita Nocerino, Carmen Di Scala, Giusy Della Gatta and Margherita Di Costanzo have been equally contributed to this chapter.

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Abbreviations

BLG	β-lactoglobulin
CMA	cow's milk allergy
EHCF	extensively hydrolyzed casein formula
FA	food allergy
LAB	lactic acid bacteria
LGG	*Lactobacillus rhamnosus* GG
OIT	oral food immunotherapy
OVA	ovalbumin
PBMCs	peripheral blood mononuclear cells

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REVIEW
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Gut Microbiome as Target for Innovative Strategies Against Food Allergy

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The dramatic increase in food allergy prevalence and severity globally requires effective strategies. Food allergy derives from a defect in immune tolerance mechanisms. Immune tolerance is modulated by gut microbiota function and structure, and microbiome alterations (dysbiosis) have a pivotal role in the development of food allergy. Environmental factors, including a low-fiber/high-fat diet, cesarean delivery, antiseptic agents, lack of breastfeeding, and drugs can induce gut microbiome dysbiosis, and have been associated with food allergy. New experimental tools and technologies have provided information regarding the role of metabolites generated from dietary nutrients and selected probiotic strains that could act on immune tolerance mechanisms. The mechanisms are multiple and still not completely defined. Increasing evidence has provided useful information on optimal bacterial species/strains, dosage, and timing for intervention. The increased knowledge of the crucial role played by nutrients and gut microbiota-derived metabolites is opening the way to a post-biotic approach in the stimulation of immune tolerance through epigenetic regulation. This review focused on the potential role of gut microbiome as the target for innovative strategies against food allergy.

Keywords: immune tolerance, gut microbiota, mediterranean diet, dysbiosis, probiotics, gut microbiota metabolites, short chain fatty acids, butyrate

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INTRODUCTION

The Changing Scenario of Food Allergy

Food allergy (FA) is one of the most common allergic disorders in the pediatric age, and it has been considered as a global health problem, particularly in industrialized world (1). During the last two decades, studies have suggested that the epidemiology of FA has shown a dramatic increase in the prevalence, severity of clinical manifestations and risk of persistence into later ages, leading to an increase in medical visits, hospital admissions, treatments, burden of care on families, and economic impact, with an increase of costs for the families and healthcare system (2–4). According to the most recent epidemiological data, time trend analysis showed up to a 7-fold increase in hospital admissions for food severe allergic reactions in children in the

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Randomized controlled trial on the influence of dietary intervention on epigenetic mechanisms in children with cow's milk allergy: the EPICMA study

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Epigenetic mechanisms could drive the disease course of cow's milk allergy (CMA) and formula choice could modulate these pathways. We compared the effect of two different dietary approaches on epigenetic mechanisms in CMA children. Randomized controlled trial on IgE-mediated CMA children receiving a 12-month treatment with extensively hydrolyzed casein formula containing the probiotic *L.rhamnosus* GG (EHCF + LGG) or with soy formula (SF). At the baseline, after 6 and 12 months of treatment *FoxP3* methylation rate and its expression in CD4+ T cells were assessed. At same study points IL-4, IL-5, IL-10, and IFN-γ methylation rate, expression and serum concentration, miRNAs expression were also investigated. 20 children (10/group) were evaluated. Baseline demographic, clinical and epigenetic features were similar in the two study groups. At 6 and 12 months, EHCF + LGG group showed a significant increase in *FoxP3* demethylation rate compared to SF group. At the same study points, EHCF + LGG group presented a higher increase in IL-4 and IL-5 and a higher reduction in IL-10 and IFN-γ DNA methylation rate compared to SF group. A different modulation of miR-155, -146a, -128 and -193a expression was observed in EHCF + LGG vs. SF. Dietary intervention could exert a different epigenetic modulation on the immune system in CMA children.

Cow's milk allergy (CMA) is one of the most frequent food allergies (FAs) in the pediatric age[1], and is the leading cause of food-induced anaphylaxis in Italian children[2]. CMA prevalence and persistence have been on the rise under the pressure of gene environment interactions leading to immune system dysfunction, mediated at least in part by epigenetic mechanisms[3,4]. Preliminary cross-sectional pilot studies suggest that epigenetic mechanisms drive CMA disease course[5–7]. Immune tolerance is defined as the active suppression of specific immune responses to dietary antigens in the gastrointestinal tract. A subset of regulatory dendritic cells (DCs), expressing CD103, is responsible for delivery of antigen to the draining lymph node and induction of regulatory T cells (Tregs)[8]. Tregs play a pivotal role in immune tolerance[9]. Forkhead box P3 (*FoxP3*) is the major transcription factor that modulates the fate of Tregs[10,11]. The methylation status of FoxP3 is regulated within a highly conserved region within the Treg-specific demethylated region (TSDR), a CpG-rich region[12]. Results from a pilot study showed different *FoxP3* demethylation status comparing CMA children with active disease with those with recent evidence of immune tolerance acquisition[6]. Dietary factors exert a pivotal role in the regulation of epigenetic mechanisms[13]. We observed a significant difference in DNA methylation of T helper (Th)1/Th2 cytokine genes in children who

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The therapeutic efficacy of *Bifidobacterium animalis* subsp. *lactis* BB-12® in infant colic: A randomised, double blind, placebo-controlled trial

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Summary

Background: The pathogenesis of infant colic is poorly defined. Gut microbiota seems to be involved, supporting the potential therapeutic role of probiotics.

Aims: To assess the rate of infants with a reduction of ≥50% of mean daily crying duration after 28 days of intervention with the probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12® (BB-12). Secondary outcomes were daily number of crying episodes, sleeping time, number of bowel movements and stool consistency.

Methods: Randomized controlled trial (RCT) on otherwise healthy exclusively breast-fed infants with infant colic randomly allocated to receive BB-12 (1×10^9 CFU/day) or placebo for 28 days. Gut microbiota structure and butyrate, beta-defensin-2 (HBD-2), cathelicidin (LL-37), secretory IgA (sIgA) and faecal calprotectin levels were assessed.

Results: Eighty infants were randomised, 40/group. The rate of infants with reduction of ≥50% of mean daily crying duration was higher in infants treated with BB-12, starting from the end of 2nd week. No infant relapsed when treatment was stopped. The mean number of crying episodes decreased in both groups, but with a higher effect in BB-12 group (−4.7 ± 3.4 vs −2.3 ± 2.2, $P < 0.05$). Mean daily stool frequency decreased in both groups but the effect was significantly higher in the BB-12 group; stool consistency was similar between the two groups. An increase in *Bifidobacterium* abundance (with significant correlation with crying time reduction), butyrate and HBD-2, LL-37, sIgA levels associated with a decrease in faecal calprotectin level were observed in the BB-12 group.

Conclusions: Supplementation with BB-12 is effective in managing infant colic. The effect could derive from immune and non-immune mechanisms associated with a modulation of gut microbiota structure and function.

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Protective action of *Bacillus clausii* probiotic strains in an in vitro model of *Rotavirus* infection

Lorella Paparo[1,2,6], Lorella Tripodi[2,6], Cristina Bruno[2], Laura Pisapia[1,2], Carla Damiano[1], Lucio Pastore[2,3,5] & Roberto Berni Canani[1,2,4,5]✉

Rotavirus **is the most common cause of acute gastroenteritis (AGE) in young children. *Bacillus clausii* (*B. clausii*) is a spore-forming probiotic that is able to colonize the gut. A mixture of four *B. clausii* strains (O/C, T, SIN and N/R) is commonly used for the treatment of AGE, and it has been demonstrated that it can reduce the duration and severity of diarrhea in children with AGE. Few studies have sought to characterize the mechanisms responsible for such beneficial effects. Intestinal effects of probiotics are likely to be strain-specific. We conducted a series of in vitro experiments investigating the activities of this mixture of *B. clausii* strains on biomarkers of mucosal barrier integrity and immune function in a cellular model of *Rotavirus* infection. *B. clausii* protected enterocytes against *Rotavirus*-induced decrease in trans-epithelial electrical resistance, and up-regulated expression of mucin 5AC and tight junction proteins (occludin and zonula occludens-1), all of which are important for effective mucosal barrier function. *B. clausii* also inhibited reactive oxygen species production and release of pro-inflammatory cytokines (interleukin-8 and interferon-β) in *Rotavirus*-infected cells, and down-regulated pro-inflammatory Toll-like receptor 3 pathway gene expression. Such mechanisms likely contributed to the observed protective effects of *B. clausii* against reduced cell proliferation and increased apoptosis in *Rotavirus*-infected enterocytes.**

Acute gastroenteritis (AGE), defined as sudden-onset diarrhea that is unrelated to chronic disease, with or without nausea, vomiting, fever or abdominal pain, is disproportionately common among young children[1,2]. *Rotavirus* (RV) is the most common cause of AGE and the leading cause of AGE-associated mortality in children younger than 5 years of age[2–6]. In 2016, more than 258 million episodes of diarrhea and approximately 1.5 million hospitalizations and 128,500 deaths in children younger than 5 years were attributable to RV infection globally[2,7]. The highest rates of RV-associated mortality have been reported in sub-Saharan Africa, Southeast Asia, and South Asia[7]. The high cost of RV vaccination precludes its widespread use in such low-income settings[8]. However, even in developed countries, AGE remains a considerable burden, despite the implementation of RV vaccination programs[7]. For example, routine RV vaccination was introduced in 2006 in the US, but there were 70,553 AGE-associated hospital admissions, about 20,000 due to RV infection, among US children younger than 5 years in 2013, which were associated with direct costs of more than US $226 million[9].

Probiotics are living microorganisms that, when administered in adequate amounts, confer a health benefit on the host after colonizing the gut, and can help to prevent and treat AGE by supporting a healthy gut and immune system[10,11]. Short- and long-term beneficial effects of probiotics on the gut are the result of a range of mechanisms, including competitive exclusion and direct antagonism of gut pathogens, stimulation of host mucosal immune mechanisms, and reconstitution and enhancement of intestinal barrier function[3,11,12]. However, not all such beneficial effects can be ascribed to probiotics as a general class, as effects occurring at the intestinal or extra-intestinal level are likely to be strain-specific[11].

Bacillus clausii (*B. clausii*) is a rod-shaped, spore-forming, aerobic, Gram-positive probiotic bacterium that is acid resistant and able to colonize the gut[13–16]. Data suggest that a mixture of four *B. clausii* probiotic strains (O/C,

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Editorial: interventions in infantile colic – can efficacy be attributed to treatment or to time? Authors' reply

We thank Dr Iacovou[1] for the interest in our paper describing the therapeutic efficacy of *Bifidobacterium animalis* subsp *lactis* BB-12 (BB-12) in infantile colic.[2] We agree with Dr Iacovou, the age at enrolment is crucial for randomized controlled trials (RCTs) on infantile colic, which usually peaks at around 6 weeks of age with progressive symptoms resolution by 3-6 months of age.[3,4] For these reasons, with the aim to reduce the risk of bias related to the self-limited nature of infantile colic, we planned to evaluate in our 4-week trial only infants aged <7 w. A similar design was adopted by others.[5-7] All infants evaluated in our RCT were aged <6 weeks at enrolment, and we observed significant impact of BB-12 on daily crying duration and the number of crying episodes starting from the second week of therapy (before the age of 3 months in all subjects).

Actually, dietary intervention for the lactating mothers has not been included in the management of infantile colic because the intervention could have negative impact on maternal-infant interaction and on the longer term continuation of breastfeeding.[8] However, recent data suggest possible benefit deriving from reduced FODMAP content in maternal diet. If confirmed by future studies, this strategy could change that opinion.[9] In our study, the possible influence of maternal dietary factors or changes in dietary habits was assessed by analyzing data from 7-day food diary collected at baseline and during the last week of treatment. No dietary changes were observed during the study.

Regarding the diagnosis of infantile colic, it was defined according to the best diagnostic criteria available when the trial was designed (the Rome III Criteria: paroxysms of irritability, fussing or crying that start and stop without obvious cause; with episodes lasting ≥3 hours per day and occurring at least 3 days per week for at least 1 week; and no failure to thrive).[10] In Figure S2, we reported just one of the three symptoms that should be considered for the diagnosis of infantile colic and, as described in the text, at baseline the difference between the two study groups was not significant.

Dr Iacovou suggested to use a new score to assess stool pattern, but this method only became available in November 2018 when our RCT was already completed. Moreover, as stated in the text, infants did not take pre/pro/synbiotics, anti-colic medications or supplementation with other nutritious fluids during the study.

In a well-defined study population of colicky infants, we investigated simultaneously clinical outcomes and potential mechanisms of action of a well-characterized probiotic strain. We think that data on the good safety profile of the probiotic strain, impressive clinical results together with modulation of gut inflammation and microbiota structure and function justify the statement of 'compelling evidence' for the efficacy of BB-12 in the treatment of infantile colic.

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This article is linked to Nocerino et al and Iacovou papers. To view these articles, visit https://doi.org/10.1111/apt.15561 and https://doi.org/10.1111/apt.15599.

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Tolerogenic Effect Elicited by Protein Fraction Derived From Different Formulas for Dietary Treatment of Cow's Milk Allergy in Human Cells

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Several formulas are available for the dietary treatment of cow's milk allergy (CMA). Clinical data suggest potentially different effect on immune tolerance elicited by these formulas. We aimed to comparatively evaluate the tolerogenic effect elicited by the protein fraction of different formulas available for the dietary treatment of CMA. Five formulas were compared: extensively hydrolyzed whey formula (EHWF), extensively hydrolyzed casein formula (EHCF), hydrolyzed rice formula (HRF), soy formula (SF), and amino acid-based formula (AAF). The formulas were reconstituted in water according to the manufacturer's instructions and subjected to an *in vitro* infant gut simulated digestion using a sequential gastric and duodenal static model. Protein fraction was then purified and used for the experiments on non-immune and immune components of tolerance network in human enterocytes and in peripheral mononuclear blood cells (PBMCs). We assessed epithelial layer permeability and tight junction proteins (occludin and zonula occludens-1, ZO-1), mucin 5AC, IL-33, and thymic stromal lymphopoietin (TSLP) in human enterocytes. In addition, Th1/Th2 cytokine response and Tregs activation were investigated in PBMCs from IgE-mediated CMA infants. EHCF-derived protein fraction positively modulated the expression of gut barrier components (mucin 5AC, occludin and ZO-1) in human enterocytes, while SF was able to stimulate the expression of occludin only. EHWF and HRF protein fractions elicited a significant increase in TSLP production, while IL-33 release was significantly increased by HRF and SF protein fractions in human enterocytes. Only EHCF-derived protein fraction elicited an increase of the tolerogenic cytokines production (IL-10, IFN-γ) and of activated CD4+FoxP3+ Treg number, through *NFAT*, *AP1*, and *Nf-Kb1* pathway. The effect paralleled with an up-regulation of *FoxP3* demethylation rate. Protein fraction from all the study formulas was unable to induce Th2 cytokines production. The results suggest a different regulatory action on tolerogenic mechanisms elicited by protein fraction from different formulas commonly used for CMA management. EHCF-derived protein fraction was able to elicit tolerogenic effect through at least in part

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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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ABSTRACT

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, VEGFβ, 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, VEGFβ, 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).

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Butyrate as a bioactive human milk protective component against food allergy

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Abstract

Background: Food allergy (FA) is a growing health problem worldwide. Effective strategies are advocated to limit the disease burden. Human milk (HM) could be considered as a protective factor against FA, but its mechanisms remain unclear. Butyrate is a gut microbiota-derived metabolite able to exert several immunomodulatory functions. We aimed to define the butyrate concentration in HM, and to see whether the butyrate concentration detected in HM is able to modulate the mechanisms of immune tolerance.

Methods: HM butyrate concentration from 109 healthy women was assessed by GS-MS. The effect of HM butyrate on tolerogenic mechanisms was assessed in *in vivo* and *in vitro* models.

Results: The median butyrate concentration in mature HM was 0.75 mM. This butyrate concentration was responsible for the maximum modulatory effects observed in all experimental models evaluated in this study. Data from mouse model show that in basal condition, butyrate up-regulated the expression of several biomarkers of gut barrier integrity, and of tolerogenic cytokines. Pretreatment with butyrate significantly reduced allergic response in three animal models of FA, with a stimulation of tolerogenic cytokines, inhibition of Th2 cytokines production and a modulation of oxidative stress. Data from human cell models show that butyrate stimulated human beta defensin-3, mucus components and tight junctions expression in human enterocytes, and IL-10, IFN-γ and FoxP3 expression through epigenetic mechanisms in PBMCs from FA children. Furthermore, it promoted the precursors of M2 macrophages, DCs and regulatory T cells.

Conclusion: The study's findings suggest the importance of butyrate as a pivotal HM compound able to protect against FA.

Lorella Paparo and Rita Nocerino equally contributed to this paper.

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Article

Phenylalanine Butyramide Is a New Cosmetic Ingredient with Soothing and Anti-Reddening Potential

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Abstract: Human skin is colonized by diverse commensal microbes, making up the skin microbiota (SM), contributing to skin integrity and homeostasis. Many of the beneficial effects aroused by the SM are exerted by microbial metabolites such as short-chain fatty acids (SCFAs), including butyric acid. The SCFAs can be used in cosmetic formulations against skin diseases to protect SM by preserving and/or restoring their natural balance. Unpleasant sensorial properties and unfavorable physico-chemical properties of butyrate strongly limit its cosmetic use. In contrast, some butyrate derivatives, including phenylalanine butyramide ($C_{13}H_{18}N_2O_2$, FBA), a solid form of butyric acid, are odorless while retaining the pharmacokinetic properties and safety profile of butyric acid. This study assessed the FBA's permeation across the skin and its soothing and anti-reddening potential to estimate its cosmetic application. The dosage method used to estimate FBA's levels was validated to be sure of analytical results. The FBA diffusion tests were estimated in vitro using a Franz-type vertical diffusion cell. The soothing action was evaluated in vivo by Colorimeter CL400, measuring the erythema index. The results suggest that the FBA represents an innovative way to exploit the benefits of butyric acid in the cosmetic fields since it cannot reach the bloodstream, is odorless, and has a significative soothing action (decrease the erythema index −15.7% after 30′, and −17.8% after 60′).

Keywords: butyrate; skin microbiota; skin permeation; erythema index; soothing and anti-reddening effect

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1. Introduction

The skin is the most significant interface between the human body and the environment. Exposure to UV B (UVB, wavelength of 280–320 nm) can generate reactive oxygen species (ROS) in the skin, exposing the biological tissue to oxidative stress [1], lipid peroxidation [2], chronic inflammation [3], and DNA damage [4–6], which can result in erythema, edema, and epidermal hyperplasia [7]. The skin is colonized by diverse commensal microbes composed of bacteria, fungi, viruses, archaea, and mites as a part of a network that is defined as SM that mediates essential physiological and pathological processes [8] and ensures its homeostasis contributing to the skin barrier function [9,10]. The skin microbes release enzymes involved in the stratum corneum renewal and desquamation process (proteases), lipidic film surface breakdown (lipases), and able to degrade urea (ureases). Moreover, the microbiota produces bacteriocins, biofilms [11,12], antimicrobial peptides [13,14], and indoles that inhibit many molds and yeasts [15]. The microbial

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BRIEF RESEARCH REPORT
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Age-Related Differences in the Expression of Most Relevant Mediators of SARS-CoV-2 Infection in Human Respiratory and Gastrointestinal Tract

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Background: Clinical features of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection seem to differ in children compared to that in adults. It has been hypothesized that the lower clinical severity in children could be influenced by differential expression of the main host functional receptor to SARS-CoV-2, the angiotensin-converting enzyme 2 (ACE2), but data are still conflicting. To explore the origin of age-dependent clinical features of coronavirus disease 2019 (COVID-19), we comparatively evaluated the expression in children and adult subjects of the most relevant mediators of the SARS-CoV-2 infection: ACE2, angiotensin-converting enzyme 1 (ACE1), transmembrane serine protease-2 (TMPRSS2), and neuropilin-1 (NRP1), at upper respiratory tract and small intestine level.

Methods: The expression of *ACE2*, *ACE1*, *TMPRSS2*, and *NRP1* in nasal epithelium and in small intestine epithelium was investigated by quantitative real-time PCR analysis.

Results: We found no differences in *ACE2*, *ACE1*, and *TMPRSS2* expression in the nasal epithelium comparing children and adult subjects. In contrast, nasal epithelium *NRP1* expression was lower in children compared to that in adults. Intestinal *ACE2* expression was higher in children compared to that in adults, whereas intestinal *ACE1* expression was higher in adults. Intestinal *TMPRSS2* and *NRP1* expression was similar comparing children and adult subjects.

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Article

A New Butyrate Releaser Exerts a Protective Action against SARS-CoV-2 Infection in Human Intestine

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Abstract: Butyrate is a major gut microbiome metabolite that regulates several defense mechanisms against infectious diseases. Alterations in the gut microbiome, leading to reduced butyrate production, have been reported in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. A new butyrate releaser, useful for all the known applications of butyrate, presenting physiochemical characteristics suitable for easy oral administration, (*N*-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA), has been recently developed. We investigated the protective action of FBA against SARS-CoV-2 infection in the human small intestine and enterocytes. Relevant aspects of SARS-CoV-2 infection were assessed: infectivity, host functional receptor angiotensin-converting enzyme-2 (*ACE2*), transmembrane protease serine 2 (*TMPRSS2*), neuropilin-1 (*NRP1*), pro-inflammatory cytokines expression, genes involved in the antiviral response and the activation of Nf-kB nuclear factor (erythroid-derived 2-like) 2 (Nfr2) pathways. We found that FBA positively modulates the crucial aspects of the infection in small intestinal biopsies and human enterocytes, reducing the expression of *ACE2*, *TMPRSS2* and *NRP1*, pro-inflammatory cytokines interleukin *(IL)-15*, monocyte chemoattractant protein-1 (*MCP-1*) and *TNF-α*, and regulating several genes involved in antiviral pathways. FBA was also able to reduce the number of SARS-CoV-2-infected cells, and *ACE2*, *TMPRSS2* and *NRP1* expression. Lastly, through the inhibition of Nf-kB and the up-regulation of Nfr2, it was also able to reduce the expression of pro-inflammatory cytokines *IL-15*, *MCP-1* and *TNF-α* in human enterocytes. The new butyrate releaser, FBA, exerts a preventive action against SARS-CoV-2 infection. It could be considered as an innovative strategy to limit COVID-19.

Keywords: COVID-19; viral infection; transmembrane protease serine 2; angiotensin-converting enzyme-2; intestinal models

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1. Introduction

There is a global pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. The gastrointestinal tract is a major target organ for SARS-CoV-2 infection, with many patients presenting with gastrointestinal symptoms, such as nausea/vomiting, diarrhea and abdominal pain. These symptoms are even more frequent in severe COVID-19 patients [2,3].

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Article

Baseline Concentrations of Various Immune Biomarkers Determine Their Increase after Consumption of a Postbiotic Based on Cow's Milk Fermented with *Lactobacillus paracasei* CBA L74 in Both Newborns and Young Children

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† Dick van Olderen passed away on 7 July 2021.

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Abstract: Intake of a postbiotic product can support immunity depending on specific conditions of the consumer. The present study evaluates the potential impact of baseline values on the change of various immune factors (α-defensin, β-defensin, cathelicidin, and secretory IgA) after three months of consumption of a postbiotic based on cow's milk fermented with *Lactobacillus paracasei* CBA L74 in a young population. For the analysis, raw data of three studies were used in a multivariate analysis applying confounding factors. One study in newborns demonstrated that intake of the postbiotic yielded an increase in the concentrations of α-defensin and secretory IgA (at least $p < 0.02$), while for all factors, except β-defensin, the higher the baseline values the lower the increase (at least $p < 0.002$). Two combined studies in young children (aged 1–4 years) showed an increase in the concentration of all factors after intake of the postbiotic (at least $p < 0.003$), but now showing the higher the baseline values the higher the increase after three months (at least $p < 0.02$) in only the postbiotic group. It is concluded that consumption of the postbiotic leads to a baseline- and age-dependent increase in the concentrations of the immune factors under study in both newborns and young children. It is hypothesized that maturation of the immune system leads to different effects on optimizing host defense factors via this postbiotic intake.

Keywords: probiotic; postbiotic; human health; beneficial characteristics; *Lactobacillus paracasei* CBA L74; α-defensin; β-defensin; cathelicidin; secretory IgA

1. Introduction

Pre- and probiotics in food applications are well known for their beneficial effects for human health [1,2]. A relatively novel development in this area is the application of postbiotics [3]. The concept of postbiotics is defined as all gut microbiome (GM)-derived substances that confer a beneficial effect to the host and do not meet the pre-/probiotic definition. These GM-derived substances can comprise both microbial compounds and microbial metabolism (synthesis of metabolites and products from microbial fermentation of several foods) [4,5]. Microbial compounds include peptidoglycans, polysaccharides,