NUTRACEUTICALS: NEW PERSPECTIVES AND APPROACHES IN HUMAN HEALTH AND DISEASE

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Editorial: Nutraceuticals: New perspectives and approaches in human health and disease

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Editorial on the Research Topic
Nutraceuticals: New perspectives and approaches in human health and disease

A nutraceutical is defined as a "food (or a part of food) that provides medical or health benefits, including the prevention and/or treatment of a disease" (Das et al., 2012). The term is applied to all components, or active ingredients, derived from foods of animal, vegetable or microbial origin, such as milk proteins, herbal products, phytochemicals and probiotics, that, other than nutrition, exhibit beneficial effects for human health. Recently, the interest in nutraceuticals is growing as most of them possess multiple therapeutic properties, including anti-microbial, anti-oxidant, anti-inflammatory and anti-cancer ones. Importantly, these products are usually recognized as "safe" and less likely to exert side effects.

Most nutraceutical intake occurs orally, and bioavailability varies greatly depending on several factors, such as physico-chemical properties, pH resistance and absorption rate. One of the main challenges for nutraceutical research relies on the effort to maximize both source quality and body delivery. Indeed, in the last decades, the optimization of nutraceutical formulations has greatly implemented the manufacturing and marketing in both pharmaceutical and food industry. On the other hand, food products for nutritional purposes are not regulated like licensed medicines and scientific research is often misinterpreted or overstretched for commercial interests. Therefore, it is imperative to re-evaluate basic research, concerning structure-function relationship, molecular interactions, activation/inhibition of cellular signaling, to reveal the actual (in) efficacy of such products on human health and disease.

A total of 11 manuscripts were published in this Research Topic including six reviews and five original research articles.

Two reviews have highlighted the role of colostrum and its components in human health. In the manuscript by Kaplan et al., the Authors critically analyze the industrial

processes that can influence the composition and the nutritional values of bovine colostrum, and introduce new advanced technologies in the field aimed at preserving the quality of the end-products (Kaplan et al.). In the review by Ramirez-Rico et al., lactoferrin (Lf), an iron-binding glycoprotein primarily found in milk, is presented as a promising compound able to regulate the inflammatory response and maintain gut homeostasis, thus counteracting and potentially preventing colorectal cancer as well reducing, as an adjuvant, side effects of chemotherapy (Ramirez-Rico et al.).

Pharmaceutical interest in the human intestinal microbiota has increased considerably, in the light of studies linking the human intestinal microbial ecology to an increasing number of non-communicable diseases. Many efforts in modulating gut microbiota have been made by using probiotics, prebiotics and, recently, postbiotics. The review by Spisni et al. summarizes all *in vitro*, *in vivo* and clinical studies in such a field, demonstrating the efficacy of these preparations in intestinal microbial homeostasis, by selectively acting on pathobionts, without altering the portion of health-associated commensals (Spisni et al.). In the study by Brunelli et al., human cell line models were used to assess the potential capacity of AminoAlta™ probiotic formulation (AApf) to protect from the physiological damages that an intense physical activity may cause. The obtained results revealed that the bacteria present in the AApf have the ability to reduce trans-epithelial permeability and decrease NF-κB signaling in Caco-2 cells under inflammatory stimulation as well as to trigger in macrophagic THP-1 cells the expression of cytokines, such as IL-1β, IL-6, and TNF-α, which typically intervene in counteracting bacterial and viral infections (Brunelli et al.). Some genetically modified bacteria, including probiotics, represent attractive vehicles for oral or nasal mucosal delivery of therapeutic molecules. It is the case of Suvorov et al. who present a study demonstrating the construction of the novel SARS-CoV-2 vaccine candidate employing the gene fragment of S1 SARS-CoV-2 gene. Expression data allowed to consider such genetically modified probiotic strain as an interesting candidate for vaccine against SARS-CoV-2 (Suvorov et al.). Of note, metabolites produced by the intestinal microbiota have shown to play a role in human health. In the review by Amiri et al., a potentially therapeutic role for butyrate, a bacterial secondary metabolite, in cardiovascular diseases and the mechanisms and pathways involved in the cardio-protective effects were deeply analyzed. In summary, the Authors indicate that although butyrate exhibits a wide variety of biological activities towards different cell and biochemical pathways, such as energy homeostasis, glucose and lipid metabolism, inflammation, oxidative stress and neural signaling, it remains unclear whether these findings are clinically relevant (Amiri et al.). Also Vitamin K2-7, a form of vitamin K produced by intestinal bacteria, has been demonstrated to exert health-beneficial effects in different pathological conditions, such as osteoporosis, cardiovascular disease, inflammation, cancer, Alzheimer's disease, diabetes and peripheral neuropathy, as reviewed by Jadhav et al.

Extra virgin olive oil (EVOO) from *Olea europaea*, a cornerstone in the Mediterranean diet, is well known for its nutritional and health properties, especially for prevention of cardiovascular diseases and metabolic disorders. In the paper by Di Pietro et al., the antimicrobial activity of different green extra virgin olive oil-based formulations in natural deep eutectic solvents (NaDESs) was demonstrated. Specifically, the EVOO extract showed the highest antibacterial activity against several clinical strains of *Staphylococcus aureus*, whereas oleacein in was the most effective toward various clinical strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (Di Pietro et al.). Another biologically active nutraceutical from plants is resveratrol. The study by Modzelewska et al. provides the first observation that resveratrol exerts relaxant effects in human gastric muscle strips by activating calcium-activated potassium (BK_{Ca}) channels independently of nitric oxide signaling pathways, opening new frontiers in resveratrol applications as in the treatment of gastrointestinal dyspepsia and other gastric hypermotility disorders (Modzelewska et al.).

The effect of natural products on hepatic fibrosis has been reviewed by Shan et al., highlighting the potential combination of different drugs, both of natural and recombinant origin, which have shown advantages of improving efficacy and reducing toxicity in clinical studies. Within this frame, the study by Jiang et al. has demonstrated the efficacy of new antioxidant compounds in ameliorating liver dysfunction via reducing iron in a murine model of thioacetamide-induced acute liver injury. In particular, along with the reduction of hepatic iron accumulation, the decrease of serum ALT, AST and LDH levels as well as the physiological rebalance of iron-proteins expression were observed (Jiang et al.).

The Research Topic "Nutraceuticals: New Perspectives and Approaches in Human Health and Disease" was effective in putting together new and promising studies on the subject, highlighting the potential application of natural products in current and future pharmacological research, as actual alternative or complement to standard pharmacological therapy.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Das, L., Bhaumik, E., Raychaudhuri, U., and Chakraborty, R. (2012). Role of nutraceuticals in human health. *J. Food Sci. Technol.* 49, 173–183. doi:10.1007/s13197-011-0269-4

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Production of Bovine Colostrum for Human Consumption to Improve Health

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Colostrum contains all essential nutrients for the neonate during the first days of life, with impacts that continue far beyond these first days. Bovine colostrum has been used for human consumption due to the high concentrations of bioactive proteins, vitamins, minerals, growth factors, as well as free and conjugated oligosaccharides. Processes involved in the preparation of bovine colostrum for human consumption play a pivotal role in preserving and maintaining the activity of the bioactive molecules. As bovine colostrum is a multifunctional food that offers a myriad of benefits for human health, assessing the main processes used in preparing it with both advantages and disadvantages is a crucial point to discuss. We discuss major processes effects for colostrum production on the nutritional value, some advanced technologies to preserve processed bovine colostrum and the end-product forms consumed by humans whether as dairy products or dietary supplements.

Keywords: bovine colostrum, immunoglobulins, thermal processing, drying methods, nanotechnology, liposomal technology, human consumption

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1 INTRODUCTION

Colostrum is the first fluid secreted by mammals for the first few days after parturition (Marnila and Korhonen, 2002; Stelwagen et al., 2009; Godhia and Patel, 2013). This food provides the initial supply of vital nutrients for neonates and plays a crucial part in the nutrition, protection, development, and immunological defense of the newborn (Rasmussen et al., 2016; Juhl et al., 2018). In the absence or limited availability of human milk, bovine colostrum is widely used as an alternative source for infants (Li et al., 2017). As newborn calves do not have an active adaptive immune system of their own, the high concentration of antibodies in colostrum, immunoglobulins, have a major impact on priming the calf's immune system. The small intestine of calves is permeable for the passive transfer of colostrum immunoglobulins through the intestinal wall for only a limited time. The potential for this passive transfer decreases in the first 6–12 h postnatal and becomes impermeable to immunoglobulins by about 24–48 h after birth. Therefore, it is vital to provide colostrum as soon as possible after birth to calves (Sangild, 2003; Baintner, 2007). Even though calves develop a gut closure that stops immunity transfer from mother, colostrum is produced in excess for several days (Marnila and Korhonen, 2002; Stelwagen et al., 2009; Godhia and Patel, 2013). Moreover,

bovine colostrum comprises only 0.5% of a bovine's milk output in a year, but this is far more than the calf's requirements (Oyeniyi and Hunter, 1978; Scammell, 2001). For this reason, excess colostrum has a high potential for use in various industrial-scale applications (Alexieva et al., 2011).

Bovine colostrum is comprised of essential nutrients such as proteins, fats, vitamins, and minerals. Additionally, bovine colostrum contains high levels of bioactive compounds including oligosaccharides, immunoglobulins, lactoferrin, and lysozyme (Kehoe et al., 2007), which have well-characterized roles in providing passive immunity, antimicrobial protection, and shaping the development of the gastrointestinal system in the early life of calves (Playford et al., 2001; Elfstrand et al., 2002). In addition to bovine colostrum's benefits on the health of the calves, it also improves human health in that it enhances the immune defense and gastrointestinal health of humans by its bioactive components (Arslan et al., 2021). The link between consumption of bovine colostrum by humans and the immunity defense has been investigated for long years to better understand bovine colostrum's immunological activity. Several studies have already shown that bovine colostrum has been used in clinics and therapeutical applications (Godhia and Patel, 2013; Gephart and Weller, 2014; Dzik et al., 2017). Oral intake of bovine colostrum, for instance, impacts intestinal immunity, which is resulted from an increased concentration of natural killers, lymphocytes (Hurley and Theil, 2011; Wong et al., 2014). IgG in bovine colostrum is also able to alter innate and adaptive immunity by binding to pathogens and human Fcγ R receptors which leads to phagocytosis and defense (Hessell et al., 2007).

Bovine colostrum, furthermore, is considered to be an effective option for passive immunity with its rich IgG content against a serious disease. The vaccination of cows against disease-causing pathogens before taking their colostrum samples can enhance the specificity of IgG content. IgG derived from hyperimmune bovine, in turn, can bind to the virus directly and hinder the binding of pathogens to intestinal epithelial cells (Jawhara, 2020). Regarding severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes a serious respiratory disease called coronavirus disease 19 (COVID-19) in humans, the specificity of IgG in colostrum against this virus may increase by the vaccination of cows and when people consume this hyperimmune colostrum, they may also be protected in short-term. In a clinical study, a human polyclonal IgG antibody which is derived from cattle vaccinated against MERS is considered safe and tolerable up to 50 mg/kg in healthy individuals (Luke et al., 2016; Beigel et al., 2018).

Bovine colostrum is also frequently preferred by athletes who do both endurance and resistance exercises since it offers high nutritional value. Athletes consider keeping themselves physically and physiologically at the highest level in a highly competitive environment. For this, in addition to nutrition programs, they mostly use a variety of supplements. Among these supplement products, bovine colostrum is also located a natural and healthy food source. Various studies have been shown that bovine colostrum impacts on the immune system (Pyne et al., 2015; Jones and Davison, 2019; Skarpańska-Stejnborn et al., 2020) and muscle development (Antonio et al., 2001) in athletes. Studies have shown that the use of colostrum in athletes is effective in hypertrophy, increase in muscle strength, and decrease in body fat (Antonio et al., 2001). Bovine colostrum, therefore, contributes to the prolongation of exercise duration by increasing the muscle buffering capacity during high-intensity exercises due to its rich IGF-1 content (Główka et al., 2019). For these reasons, bovine colostrum has been proposed as a way to improve human health in a variety of dietary supplements, dairy products, and nutraceuticals as a result of its bioactive potential. There are various preserved forms of bovine colostrum for human consumption as powder, liquid, tables, or capsules (Bartkiene et al., 2018; Silva et al., 2019). However, when considering the processes involved in preparing it for human consumption, the preservation and protection of the significant bioactive factors in bovine colostrum is critical (Phipps et al., 2016; Sotudeh et al., 2019). Thermal treatment and drying methods are some of the processes used to produce different forms of bovine colostrum, and each has advantages and disadvantages depending on the intended final application (Jay, 1992; Godden et al., 2006; Sotudeh et al., 2019). Therefore, the main aim of this review is to discuss the effects of thermal treatment and drying processes on the nutritional value of bovine colostrum on human consumption, as well as advanced technologies (e.g., nanotechnology and liposomal technology) to preserve processed bovine colostrum for human consumption. Moreover, end-products consumed by humans such as dietary supplements and dairy products are also discussed.

2 THERMAL TREATMENT METHODS

The preservation of high-quality colostrum that is free from microbial contamination is one of the main challenges to producing colostrum-based products for human consumption (Phipps et al., 2016; Sotudeh et al., 2018). There are several pathogens that cause contamination of colostrum, but contamination is principally caused by *Mycobacterium avium ssp. Paratuberculosis, Mycoplasma spp., Escherichia coli* (*E. coli*), *and Salmonella spp.* (Streeter et al., 1995; González and Wilson, 2003; Houser et al., 2008). These pathogens often originate from the mammary glands and contaminate colostrum during milking, and can grow during storage, creating a potential risk for the final product (Fecteau et al., 2002; McGuirk and Collins, 2004). In calves, it may interfere with the passive absorption of colostrum antibodies, but in colostrum for human consumption, it can affects the protein and colostrum-based products' quality (Houser et al., 2008). Therefore, effective elimination of pathogens requires thermal processing, most commonly pasteurization and sterilization (Jay, 1992), to ensure high-quality colostrum production (Sotudeh et al., 2018).

2.1 Pasteurization

Pasteurization is the heat-treatment process to destroy pathogenic microorganisms such as bacteria, protozoa, molds, and yeasts in beverages and foods (Jay, 1992). Pasteurization reduces the number of viable microbial cells rather than

destroying all microorganisms (Elizondo-Salazar and Heinrichs, 2009). There are two pasteurization methods: high-temperature short-time (HTST) and low-temperature long-time (LTLT) pasteurization (Jay, 1992).

2.1.1 High-Temperature Short-Time (HTST) Pasteurization

One of the most common pasteurization methods is HTST, also known as flash pasteurization or continuous pasteurization. In HTST, colostrum is circulated and heated up to 72°C (161°F) during 15 s (s) as it passes through heated coils (Jay, 1992). If the appropriate temperature is not reached during the first circle, the fluid is discharged back into the original tank, and the circulation process is repeated. These systems are designed to then rapidly cool the colostrum to feeding or storage temperatures (Elizondo-Salazar and Heinrichs, 2009). Several studies have shown that HTST processing on bovine colostrum is sufficient to destroy many common pathogens in raw milk, such as *Mycobacterium paratuberculosis (M. paratuberculosis), Salmonella enterica spp.*, and *Mycoplasma spp.*, while others have shown that *M. paratuberculosis* is destroyed using HTST on bovine colostrum (Godden et al., 2003; Stabel et al., 2004). Stabel et al. (2004) showed that HTST pasteurization was sufficient to remove viable *M. paratuberculosis*. However, notable reductions in the quality of bovine colostrum were observed, including approximately 25% reduction in intact immunoglobulin G (IgG) concentration after HTST pasteurization. Additionally, the process significantly increases the viscosity of the fluid during or after the pasteurization process (Godden et al., 2003). Taken together, these studies suggest that HTST pasteurization is an efficient method of pathogen destruction while simultaneously ensuring the majority of bioactive molecules remain to produce high-quality colostrum-based products for human consumption (Elizondo-Salazar and Heinrichs, 2009). However, special considerations for the increase in viscosity are important for downstream applications.

Microfiltration is another process, which can be combined with HTST, as an alternative way to extend the shelf-life of dairy products (Al-Akoum et al., 2002). This process is increasingly used in the dairy industry to remove bacteria by membranes, which is also feasible in technique and economics. Microfiltration eliminates all pathogens from bovine colostrum or milk regardless of any denaturation of bioactive proteins or nutritional content (Hansen et al., 2020). It may also preserve the bioavailability of multifunctional components including peptides, antioxidants, and vitamins (Papadatos et al., 2003; Amelia and Barbano, 2013). Tetra Pak developed a process called Bactocatch which is the most common microfiltration to remove bacteria from the products. Membrane fouling is decreased significantly by a crossflow uniform transmembrane pressure in the bactocatch system (Sandblom, 1978). Microfiltered retentate and cream parts of dairy products are treated under 130°C for 4 s and combined with skimmed permeate which is almost bacteria free and the milk is processed with a minimum HTST. The bactocatch process reduces total bacteria by 2.8 logs in reports (Bindith et al., 1996; Hoffmann et al., 1996). In a study, bovine colostrum is treated by a membrane filtration process (pore size 0.1 μm). The process recovered at least 80% of the IgG and other minor whey proteins in the micro filtrate part (Piot M et al., 2004).

2.1.2 Low-Temperature Long-Time (LTLT) Pasteurization

LTLT is another thermal treatment that is also known as Holder pasteurization or batch pasteurization. LTLT pasteurization is performed when a tank or batch of colostrum is heated up to the target temperature [63°C (145°F)], for a minimum of 30 min (Jay, 1992). LTLT pasteurizers should be equipped with an agitator for allowing to homogeneous heating throughout the entire batch of fluid. Following the 30 min heat treatment, batches are quickly cooled to feeding or storage temperature. One challenge with LTLT pasteurization is the batch size of the sample and the processing time. For instance, batch size impacts on the duration of pasteurization, when sizeable batches are used for pasteurization, the processing time may take several hours. Thus, some bacteria (e.g., some *Salmonella* spp.) may survive the process.

In contrast to HTST pasteurization, LTLT pasteurization of bovine colostrum was less effective at removing bacterial contaminants. In Meylan et al. (1996), colostrum samples were inoculated with *M. paratuberculosis* and then heated to 63°C for 30 min to test whether *M. paratuberculosis* could survive pasteurization. They also examined the effect of pasteurization on IgG concentrations. *M. paratuberculosis* was not eliminated by pasteurization, but its growth was slowed. The mean IgG concentration of unpasteurized and pasteurized ones were measured as 44.4 ± 30.3 g/L and 37.2 ± 23.8 g/L, respectively, representing a fall of 12.3 ± 8.7% IgG, which was not interpreted as a hindrance for successful passive transfer of immunity to calves (Meylan et al., 1996).

In a similar study by Godden et al. (2003), the efficacy of the commercial batch pasteurization method on the concentration of IgG content and the feeding features of bovine colostrum samples was investigated. The effect of pasteurization on IgG concentrations of serum samples of calves fed unpasteurized and pasteurized colostrum was also examined. In contrast to Meylan et al. (1996), the study showed that LTLT pasteurization (63°C, 30 min) declined IgG concentration by 58.5 and 23.6% for 95-L and 57-L batches, respectively. Pasteurization of 57-L batches produced high quality (vs. 95-L) and standard or mildly thickened bovine colostrum that could be used to feed calves. When pasteurization effects on serum IgG level was analyzed, a significantly lower serum IgG concentration was found in calves fed pasteurized colostrum, relative to unpasteurized colostrum. For example, serum IgG concentrations of 40 calves fed unpasteurized colostrum (19.1 g/L) were 9.4 g/L higher than serum IgG concentrations of 55 calves that are fed pasteurized colostrum (9.7 g/L) when calves were fed 2 L colostrum at the first nutrition time. In contrast, serum IgG levels between eight calves fed unpasteurized bovine colostrum (16.1 g/L) and 20 calves fed pasteurized bovine colostrum (13.5 g/L) did not differ if calves fed 4 L at the first feeding. The study was concluded that batch pasteurization of colostrum would help preserve and maintain

colostrum (Godden et al., 2003). However, Johnson et al. (2007) reported raised IgG absorption efficiency and higher IgG level of serum samples from calves fed colostrum pasteurized for longer than 30 min. Colostrum pasteurized at 60°C, 60 min showed higher level of serum total protein (TP) and IgG, also efficiency of IgG absorption was higher in calves fed pasteurized colostrum (TP = 6.3 mg/dl; IgG = 22.3 mg/ml; apparent efficiency of absorption = 35.6%) in comparison to calves fed unpasteurized colostrum (TP = 5.9 mg/dl; IgG = 18.1 mg/ml; apparent efficiency of absorption = 26.1%). There was no significant difference found in serum Ig function and serum concentrations of IgA, IgM, vitamin E, vitamin A, cholesterol, β-carotene between fed pasteurized vs. unpasteurized colostrum at 24 h of age. Thus, the batch pasteurization of colostrum at 60°C, 60 min caused the reduced bacteria concentration in colostrum with preserving the colostrum and serum IgG concentrations (Johnson et al., 2007).

While 30 min pasteurization is the minimum for Holder pasteurization, McMartin et al. (2006) aimed to identify optimum temperature and process time of pasteurization to ensure optimal IgG concentration, the viscosity of bovine colostrum, etc., For these purposes, 50 ml bovine colostrum samples from six batches were heated at 59, 60, 61, 62, and 63°C in A Rapid Visco Analyzer (RVA). According to the results, there were no significant differences in viscosity and IgG concentrations between unpasteurized colostrum samples (73.4 ± 26.5 mg/ml) and pasteurized colostrum samples (74.5 ± 24.3 mg/ml) after colostrum samples were heated to 60°C in a RVA for 120 min. On the other hand, 50 ml colostrum samples from 30 unique batches were heated for 120 min at 60 and 63°C in a RVA to confirm the preliminary results. The second experiment showed pasteurization of colostrum at 63°C for 120 min resulted in a 34% decline in IgG level and a 33% increment in viscosity of colostrum samples (McMartin et al., 2006), suggesting that batch-to-batch variation may influence some of the findings.

In another study of S. Godden et al. (2006), bovine colostrum samples from 30-L batches were firstly inoculated with *M. bovis* (10^8 CFU/ml), *Listeria monocytogenes* (10^6 CFU/ml), *E. coli* O157:H7 (10^6 CFU/ml), *Salmonella enteritidis (S. enteritidis)* (106 CFU/ml), and *Mycobacterium avium subsp.* paratuberculosis (Map; 10^3 CFU/ml) to describe the effects of pasteurization on IgG concentration of bovine colostrum and investigate the optimum duration of heating to eliminate pathogens. After inoculation, samples were heated up to 60°C, 120 min. The subsamples of colostrum at 15 min intervals throughout the pasteurization were collected to analyze bacterial culture, measure IgG level (g/L), and the activity of antibodies [$\log_2$ (bovine viral diarrhea virus type 1 serum neutralization titer)]. As in previous studies, there was no significant difference found in IgG concentration of colostrum samples (pre: 60.5 g/L; post: 59.1 g/L) after heating colostrum to 60°C at least 120 min and no effect found in pasteurization process on mean $\log_2$ bovine viral diarrhea virus type 1 serum neutralization titer (pre = 12.3; post = 12.0). Another important result of this study; after pasteurization of colostrum at 60°C for 30 min, some pathogens such as *M. bovis, L. monocytogenes, E. coli O157:H7*, and *S. enteritidis* were not detected in samples. *M. paratuberculosis* was not observed when the colostrum was heated to 60°C for 60 min. It was concluded that heating of colostrum to 60°C for 60 min was enough to reduce the concentration of pathogens (Godden et al., 2006). When taken together with the findings of Meylan et al. (1996), the minimum time for Holder pasteurization may not be sufficient for efficient elimination of pathogens but extending this time to 60 min may improve the effect without significant losses to the bioactive potential of colostrum.

Similarly, Donahue et al. (2012) investigate the effect of pasteurization at 60°C - 60 min on total bacteria counts and IgG concentration of bovine colostrum. It was found that the pasteurization process reduced colostrum total plate counts (−2.25 $\log_{10}$) and coliform counts (−2.49 $\log_{10}$) but did not significantly change colostrum IgG concentration (Donahue et al., 2012). These results are consistent with the study by S. M. Godden et al. (2012). Godden et al. (2012) performed a randomized controlled clinical trial using 1,071 calves from six commercial dairy farms. The serum IgG concentrations were higher in calves that were fed pasteurized colostrum (18.0 ± 1.5 mg/ml) compared with calves fed unpasteurized colostrum (15.4 ± 1.5 mg/ml). In addition, calves fed pasteurized colostrum were at lower risk for illness, which was attributed to the considerable decline in colostrum total coliform count (Godden et al., 2012).

In a different study investigating the effect of pasteurized colostrum (60°C, 60 min) feeding on the colonization of microorganisms in the neonatal calves' small intestine, Malmuthuge et al. (2015) assigned three treatment groups including feeding fresh colostrum, feeding pasteurized colostrum, and control group. The total microbial colonization in the small intestine of calves was analyzed using quantitative real time-PCR and it was concluded that there was an increase in total bacterial colonization in the small intestine of calves, whether they were fed with fresh or pasteurized colostrum. In contrast, *Lactobacillus* was found in lower level in the small intestine of the pasteurized colostrum-fed group than in the control group. However, *Bifidobacterium* was detected 3.2 and 5.2-fold higher in the small intestine of feeding with pasteurized colostrum groups than the feeding with fresh colostrum and control groups. In addition to these, the colonization of *E. coli* was significantly lower in the small intestine of feeding with pasteurized colostrum group (Malmuthuge et al., 2015).

Elsohaby et al. (2015) studied the impact of different pasteurization processes (60°C and 63°C each for either 30 or 60 min) on IgG concentration of colostrum samples. Infrared (IR) spectroscopy, the reference radial immunodiffusion (RID) assay, digital and optical refractometers were used to quantify IgG concentration of colostrum. The average RID-IgG concentration of unheated and heat-treated colostrum samples was determined as 45.6 g/L and 30–48 g/L, respectively. So, there was no difference between unheated and heat-treated colostrum concentration at 60°C for 30- and 60-min, whereas the concentration of RID-IgG decreased to 31.1 and 30 g/L, after heat treatment at 63°C for 30 and 60 min, respectively. On the

other hand, the average IR-IgG concentration of unheated and heat-treated colostrum samples was measured as 44.7 g/L and 37.7–42.5 g/L, respectively and the average IR-IgG concentration of unheated and heat-treated colostrum samples regardless of temperature and time were similar. The unheated and heat-treated colostrum IgG concentrations' Brix scores were also analyzed, and no difference was found between these groups regardless of temperature and time. It was concluded that radial immunodiffusion, IR spectroscopy, and Brix refractometers could be used for measuring IgG concentration of colostrum after heat treatment at 60°C, but the decrease of colostral IgG concentration after heat treatment at 63°C could not be detected by using these methods (Elsohaby et al., 2015).

Apart from similar studies about the pasteurization effect on bovine colostrum IgG, Rafiei et al. (2019) investigated the impact of feeding pasteurized colostrum on the neonatal dairy calves' health and performance. Calves fed with pasteurized colostrum showed lower diarrhea, and pneumonia symptoms, whereas no differences in skeletal growth measurements except body barrels were observed. IgG concentration, total plate count, and passive transfer of immunity after heat treatment of colostrum at 60°C for 30 min were analyzed and there was no difference in the IgG concentration of colostrum between pasteurized and unpasteurized colostrum (57.6 mg/ml, 60.6 mg/ml, respectively). According to the results of the total plate count experiments, the pasteurized colostrum samples have lower colostrum bacterial concentrations (-2.01 CFU/ml) (Rafiei et al., 2019). Similar studies by Armengol and Fraile, (2016) concluded that feeding calves with pasteurized colostrum and milk (60°C for 60 min, 63°C, 30 min) decreased morbidity and mortality rates by 9.8 and 3.7%, respectively. The increment in bovine respiratory disease morbidity in the first year of life and diarrhea in the first 180 days of life was observed in calves who were not fed pasteurized colostrum (Armengol and Fraile, 2016).

Similar to previous studies, Hesami et al. (2020) studied the impact of different pasteurization temperatures and duration such as 60°C for 30, 60, and 90 min respectively on IgG concentration of bovine colostrum. The feeding pasteurized bovine colostrum effect on the health and performance of Holstein calves were also evaluated in this study. IgG concentrations of untreated or pasteurized colostrum at 60°C for either 30, 60, or 90 min was found to be 67.63 ± 0.08, 66.17 ± 0.08, 63.07 ± 0.08, and 59.53 ± 0.09 g/L, respectively. There was not any significant difference in the concentration of fat, protein, and ratio between fat and protein after pasteurization. It was observed that the pH of heat-treated colostrum samples increased from 6.77 ± 0.003 to 6.79 ± 0.003 but the difference was not significant statistically ($p > 0.05$). In conclusion, calves fed pasteurized colostrum had a better overall health status, whereas calves fed with unpasteurized colostrum had a lower prevalence of diarrhea-induced pathogens. During lactation, calves' health, growth characteristics, and physical performance were improved by feeding pasteurized colostrum (Hesami et al., 2020).

2.2 Sterilization

Sterilization is another thermal treatment process of heating samples for the destruction of all viable organisms. Sterilization can be achieved using several ways such as heating, ionizing radiation, high hydrostatic pressure, photodynamic effects, or other techniques. Conventional sterilization methods cause irreversible inactivation or destruction of crucial components of the microorganisms, even highly resistant bacterial spores. The ultra-high temperature process is also called UHT, one of the common methods of the sterilization process (Kumbár et al., 2015; Doyle, 2019).

2.2.1 Ultra-High Temperature (UHT)

The ultra-high temperature (UHT) process is the sterilization of dairy products at 135°C (275°F) for an extremely short period (around 2–5 s) to extend the shelf-life at room temperature. UHT treatment eliminates all viable organisms, including spores, but destroys several important components of bovine colostrum. Pasteurized bovine colostrum samples result in mostly a 15–35% reduction of IgG concentration of colostrum, while UHT treated samples almost no functional concentrations of IgG (Li-Chan et al., 1995). On the other hand, the UHT process has basic features such as its ability to process colostrum continuously, aseptic handling of the products, and stability at room temperature for up to 8 weeks without any changes (Jay, 1992).

3 DRYING METHODS

Drying methods, as thermal treatment methods, are used in the food industry, especially in the food preservation process after pasteurization. There are many studies describing processes for drying, and they are critical for the food industry. Drying facilitates long storage periods and enables the processing of colostrum without any loss of its components (Guiné, 2018). During the drying process, the water activity in food products is lowered to slow microorganism growth and the speed of chemical reactions that may degrade colostrum quality. In addition, the reduced weight of dried, powdered products and long shelf stability reduces the costs and product packaging difficulties, storage, transportation and distribution of the products (Barbosa-Canovas et al., 2005). Powder products manufactured by drying methods have several advantages, for instance, special storage conditions are not required, and less space is required. Powder products are also good food reserves for emergency situations, and they are suitable to develop new food products (Schuck et al., 2016) There are a variety of drying methods from Sun drying to freeze drying, and spray drying processes to preserve food products (**Figure 1**). Spray drying and freeze drying methods are the most common methods favored in the dairy industry (Chelack et al., 1993; Stewart et al., 2005; McMartin et al., 2019).

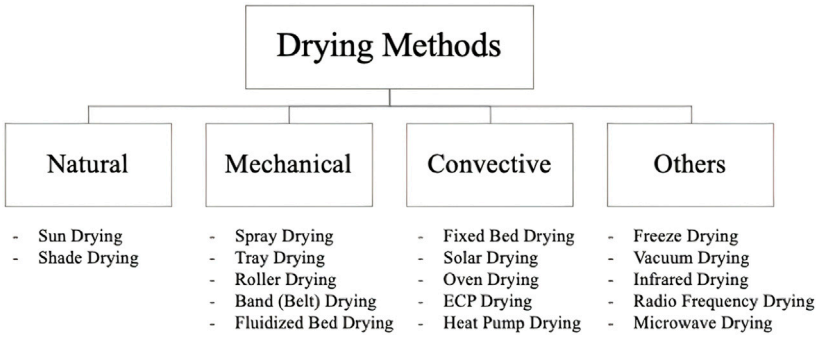

FIGURE 1 | Drying methods.

3.1 Spray Drying vs. Freeze Drying

Spray drying is a technique for producing dried powdered products based on the transformation of liquid state into a dry powdered form. In the dairy industry, spray drying is the most used technique for the manufacturing of dairy powders and some ingredients due to its low cost and wide equipment availability. The feed, which can be a solution or suspension, is sprayed into a hot dryer chamber to rapidly remove moisture. The final product is in the form of powders, agglomerates, or granules (Borad et al., 2007). The spray drying process can produce spherical shape particles with a certain particle size distribution. It has a short drying time; therefore, it is applicable to drying heat-sensitive materials. Spray drying is mainly preferred to process milk (Oakley, 1997; Schuck et al., 2016). However, spray drying of colostrum causes significantly decreased concentrations of bioactive proteins such as transforming growth factor β1 and β2, and raised protein aggregation (Støy et al., 2016). Even though it is relatively cheaper and faster, changes in morphology and particle size, aroma loss, and thermal decomposition are major disadvantages of spray drying of milk products (Gharsallaoui et al., 2007), and the loss of functional proteins in colostrum also complicates the use of spray drying for colostrum, in particular.

Freeze drying is another drying method that includes two steps. First, the product is frozen, and the suspension medium is crystallized at a low temperature. Then, the fluid is sublimed from the solid-state through the vapor phase under reduced pressure (Ciurzyńska and Lenart, 2011). Freeze dried products retain their original shape and texture, and this procedure minimizes nutrient loss due to its low processing temperature and rapid transition of frozen material from hydrate to anhydrous. Therefore, it is the most favored dehydration technique for heat-sensitive biological materials (Borad et al., 2007). Freeze drying is frequently preferred in the biotechnology sector and freeze-dried products can be stored for more than a year without loss of their biological properties (Ciurzyńska and Lenart, 2011).

Spray drying and freeze drying have some advantages such as protection of elements, which are sensitive to high temperatures, against degradation. However, freeze drying of bovine colostrum is more effective in preserving bioactivity, especially of immunoglobulins, when compared to spray drying (Elfstrand et al., 2002). Whether spray or freeze dried, colostrum powders can be used as a dietary supplement or as ingredients in new products, and evaluations of freeze and spray drying methods in terms of production speed, cost, and energy use found that while the production rate of spray drying is higher and has an overall lower cost, freeze drying is more efficient and protects immunoglobulins in colostrum better than spray drying (Chelack et al., 1993; Borad et al., 2019). Elfstrand et al. (2002) investigated the effect of such dairy processing treatments as heat treatment, ultrafiltration, and freeze drying, on the recovery of Igs (IgG, IgA, and IgM), growth factors (TGF-β2 and IGF-1) in bovine colostrum. The 30 and 25% fall in IgG and IgA, respectively, were noted in colostrum concentrate after pasteurization and freeze-drying processes compared to untreated colostrum. According to results, pasteurization caused 33% decrease of IGF-1, while a further 30% reduction was observed in freeze drying. These results indicated that the bioactive components of the bovine colostrum decreased gradually by filtration, heat treatment, and freeze drying processes (Elfstrand et al., 2002). Sotudeh et al. (2018) examined the impact of spray drying, freeze drying, and pasteurization techniques on bacterial loads and the IgG concentration of first milking postpartum bovine colostrum. All treatment groups showed an impact on the reduction of standard plate count, *E. coli* count, and total coliform count, while the spray drying, and freeze-drying were noticeably efficient at reducing microbial loads. Additionally, a reduction in IgG level was observed in all treatment groups, but a lower decrease was noted in freeze dried bovine colostrum in comparison to spray dried and pasteurized bovine colostrum. These results indicated that freeze drying and spray drying processes are functional for enhancement of the shelf-life and quality of bovine colostrum as compared to pasteurization (Sotudeh et al., 2018).

The digestibility of protein, as well as the protein structure, can be affected by heat during processing. Different drying process conditions lead to various effects on protein digestion (van Lieshout et al., 2020). Altered digestion due to modifications of proteins may affect the physiological effects of dairy proteins to the consumer. The gastrointestinal tract and the immune system can also be affected by the differently sized peptides, and various chemical modifications of these sequences (Nowak-Wegrzyn and Fiocchi, 2009). The use of spray drying in the preparation of

colostrum powder is limited, as it may cause heat-induced damage to colostrum proteins for example, causing disruption of key bioactive proteins such as lactoferrin and a loss in immunoglobulins (Bar et al., 2010; Abd El-Fattah et al., 2014; Borad et al., 2019). However, high costs in production, longer processing period, and scaling difficulties the freeze drying has limited some applications in the industry. Spray drying of colostrum generally costs 2.5 times less than freeze drying and commercial equipment is readily available (Chelack et al., 1993). Although excessive temperatures are avoided in spray drying to maintain immunoglobulin function in bovine colostrum products, it can cause the growth of pathogens and spoilage microorganisms. Therefore, spray drying conditions should be optimized for evaluating the thermal protection impact and to control microbial burdens with safety standards (Borad et al., 2019).

4 ADVANCED TECHNOLOGIES TO PRESERVE PROCESSED COLOSTRUM FOR HUMAN CONSUMPTION

Nanotechnology and liposomal technology are used to prepare bovine colostrum for human consumption with numerous advantages. Nanotechnology is a study of structures and materials on an ultra-small scale, and it has various applications in many fields from dairy industry to medicine (Chellaram et al., 2014). The nanotechnology principle is based on the modification of physicochemical properties of materials when they are reduced to the nano dimension (particles approximately 1 micron in size) (Keck et al., 2008). It provides several opportunities for the development of novel products and the expansion of food system applications including nutraceuticals, functional foods, bioactive substances, and pharmaceuticals, etc. (Samal, 2017). Even more, nanotechnology can be used to detect food pathogens, which are indicators of food quality and safety (Bott et al., 2014). This technology is increasingly used in the food market due to its advantages such as improving shelf life of products, flavor, and texture of foods. (Bajpai et al., 2012). Nanoencapsulation, which is nanometer encapsulation using films, nano dispersions, or layers, is a novel area of nanotechnology. The capsule derived by nanoencapsulation technology protects the food or active ingredients at the nanoscale. The key advantage of nanoencapsulation is the increased homogeneity, improved encapsulation efficacy as well as enhanced physical and chemical properties (Sanguansri and Augustin, 2006; Khare and Vasisht, 2014).

In the dairy industry, several bioactive proteins including whey proteins are preserved to be used as nutritional supplements. However, since most proteins are fragile, their stabilization is a crucial requirement for many applications. Nanoencapsulation technology encapsulates those bioactive proteins into nanometer-sized vesicles to prevent any denaturation or degradation that can result from proteolysis and/or dilution effects (Srinivas et al., 2010). Nanoencapsulation technology not only preserves peptide stability of proteins in dairy products, but also improves bioactive peptide delivery to target tissues (Dziuba and Dziuba, 2014). This technology provides significant advantages to avoid instability problems in the preservation of bovine colostrum and its bioactive proteins. The stability of bovine colostrum proteins, such as IgG, is crucial to preserve the bioactive potential of bovine colostrum. Bovine colostrum IgG encapsulated by 0.5% (w/v) of Tween 80, sucrose stearate, or soy protein showed increased stability of free IgG against the pH 12.0 and 2.0 environments by 33–62% and 21–56%, respectively, based on the emulsifier (Chen et al., 1999).

Liposomal technology, which is widely preferred in the pharmaceutical, cosmetic, and dairy industries, and genetic engineering, is another commonly used encapsulation method (Lipowsky and Sackmann, 1995; Jesorka and Orwar, 2008; Mozafari et al., 2008). This advanced technology uses a liposome, which is a spherical vesicle including at least one lipid bilayer and phospholipids. Liposomes can have surface ligands to ensure the binding of various ligands. These spherical vesicles can be used in many applications due to their independent biological activity and improved efficacy of encapsulated content (Umnova, 2010).

One of the liposomal technology applications is to prepare functional additives as food products for various therapeutic applications. Liposomal technology has been used to preserve bovine colostrum and increase its bioavailability for human health (Superti, 2020; Subramanian, 2021). The delivery of colostrum through the body with the reconstituted membrane phospholipids is far more effective in comparison to the colostrum without liposomal encapsulation. Furthermore, a preliminary study using liposomal encapsulated colostrum supplementation (20 g of colostrum per day) increased the bone-free lean body mass, whereas whey supplementation did not cause any increase in lean body mass (Antonio et al., 2001). With this advanced liposomal delivery system, these results indicated that there was a remarkable increase in the bioavailability of bovine colostrum. The embedding of active components into liposomes has some advantages such as enhanced bioavailability of components, controlled release of active component through the body, and prevention from any environmental factors such as temperature, oxygen, pH etc., (Mozafari et al., 2008). Thus, nanoencapsulation and liposomal technology may offer significant advantages for preserving a food product and enhancing its therapeutic activity in many food applications.

5 FORMS CONSUMED BY HUMANS (E.G., LIQUID, CHEESE, POWDER, PILL/TABLET)

In recent years, bovine colostrum has been used for human health in various dietary supplements, nutraceuticals, or in traditional medicine due to its rich nutritional content that claims to block pathogens, improve body condition, and enhance gastrointestinal health (Bartkiene et al., 2018; Juhl et al., 2018; Silva et al., 2019). Several processing treatments (thermal treatment, drying, and others) are used to prepare bovine colostrum for human

TABLE 1 | Studies evaluating the addition of bovine colostrum in dairy production.

Product	References
Yogurt	Ahmadi (2011)
Yogurt and kefir	Ahmadi (2011)
Fermented milk	Nazir et al. (2018)
Curd	Das and Seth (2017)
Khees	Poonia and Dabur (2012)
Ice cream	Mouton and Aryana (2015)
Milk-based beverages and butter	Saalfeld et al. (2008)

consumption. End-products through these processes can include liquid colostrum, a preserved dairy product, infant formula, pills, tablets, liquid, or as a powder supplement. Additionally, advanced technologies such as nanoencapsulation and liposomal technology are recently preferred to manufacture bovine colostrum for human health products (Chellaram et al., 2014).

5.1 Forms of Bovine Colostrum in Dairy Products

Liquid bovine colostrum is currently used as an ingredient in some dairy products such as yogurt, cheese, or kefir. The production of dairy foods with addition of bovine colostrum is the focus of several studies to ensure its suitability for human consumption (**Table 1**). The addition of bovine colostrum into dairy products is thought to provide an improved and healthier product for humans. In the context of yogurt production, the bovine colostrum addition represented great sensory acceptance (Ayar et al., 2016; Abdel-Ghany and Zaki, 2018). Abdel-Ghany and Zaki, (2018) and the addition of bovine colostrum increased the total protein content, total solids, IgG, lactoferrin, and minerals compared to typical yogurt (Abdel-Ghany and Zaki, 2018). Similarly, Saalfeld et al. (2008) showed sensory acceptance for milk-based beverages and butter including bovine colostrum silage, which is a kind of anaerobic fermentation methodology for excessive colostrum used for allowing milk replacement for calves (Saalfeld et al., 2008). Other traditional dairy products have also been evaluated for the addition of colostrum. Poonia and Dabur, (2012) evaluated the addition of bovine colostrum in a traditional Indian dessert known as khess (Poonia and Dabur, 2012). Das and Seth, (2017) also studied curd samples fortified with the bovine colostrum whey addition. In addition to these dairy products, bovine colostrum has also been used in cheese. For instance, a baked cheese, which is known as leipajuusto, is traditionally produced in Finland from bovine colostrum or reindeer milk. A sweet cheese called junnu is also made with bovine colostrum in southern states of India (Das and Seth, 2017).

5.2 Forms of Bovine Colostrum in Dietary Supplements

There is a noticeable increment in the number of people who consume bovine colostrum supplements with the desire to improve health (Arslan et al., 2021; Davison, 2021; Playford and Weiser, 2021). Bovine colostrum supplements can be used in several forms such as powder, liquid, tablets, or pills. The colostrum products market has different tablets containing dried colostrum, colostrum-based drinks, or dried colostrum powders. Drying processes to produce bovine colostrum supplements are appropriate for hindering protein denaturation. Specialized low heat spray drying or freeze drying are also favored to prevent these types of denaturation (Chelack et al., 1993; Stewart et al., 2005; McMartin et al., 2019).

Bovine colostrum use as a dietary supplement improves human health and wellbeing (Playford et al., 2001; Silva et al., 2019). This supplement is especially taken by athletes to improve body functions and high-intensity exercise (Mero et al., 1997, 2002). Current studies showed that the bovine colostrum supplement has some positive effects such as reducing exercise-induced muscle damage and inflammation (Kotsis et al., 2018; Jones et al., 2019). Additionally, bovine colostrum is used in the prevention against diarrhea, for improved immune system function, the treatment of AIDS-related gastrointestinal diseases, and to improve gut health (Florén et al., 2006; Patel and Rana, 2006). Further, there is some evidence that bovine colostrum helps in immune modulation, maintenance of gastrointestinal mucosal integrity, and/or promoting tissue repair (**Table 2**).

6 CONCLUSION

Food products are processed to ensure both high quality and safety while preserving their nutritional content and extending shelf-life. Practically, processing aims to destroy microbes associated with spoilage and disease, the inhibition of native enzymes, minimization of the deterioration rate of the product, and prevention of nutrients in the food. Bovine colostrum has been used in several food products and dietary supplements due to its rich nutritional content, which may confer health benefits for humans. Various processes (e.g., pasteurization, sterilization, spray drying, and freeze drying) are used to prepare bovine colostrum as appropriate for human consumption. Such processes have both advantages and disadvantages, whereas their common aim is to preserve colostrum quality associated with bioactive proteins, such as IgG, for human health. Therefore, ensuring stability of these proteins is a necessity to preserve the beneficial effect of bovine colostrum.

Thermal treatments including HTST, LTLT pasteurization, and UHT sterilization that have been investigated by several researchers for the development of bovine colostrum-based products without reducing its quality and IgG level. Thermal denaturation temperatures of different protein fractions of bovine colostrum range from 62 to 78°C whereas the immunoglobulins begin to denature at 72°C and they are fully denatured at 89°C. According to some studies, HTST pasteurization is an effective method to destroy some pathogens in colostrum but resulted in a reduction in colostrum immunoglobulins and unacceptable feeding characteristics, namely an increase in viscosity. LTLT pasteurization significantly eliminated several pathogens and reduced total bacteria and coliform counts in colostrum, while preserving IgG concentrations and the nutritional composition of colostrum. In the UHT sterilization process, bioactive proteins

TABLE 2 | Different bovine colostrum forms used in some types of diseases.

Disease	Colostrum form	Effects	Dose	References
Gastrointestinal injury by non-steroidal anti-inflammatory drugs	Powder colostrum in tablets/capsules	IGF and TGF-B linked with analgesic activity	125 ml daily	Playford et al. (2001)
Diabetes delayed injury healing by rise in the level of blood glucose	Colostrum topical cream/powder colostrum in tablets/capsules	Reduction of blood glucose	10 g daily	Kim et al. (2009)
Inflammation induced for HIV patients	Colostrum powder in the form of tablets/ capsules or liquid colostrum	Mucosal integrity, tissue repair, antimicrobial actions	10 g daily	Kaducu et al. (2011)
Ultraviolet B induced photodamage	Colostrum topical cream	Preventing damage to skin	10 g daily	Murata et al. (2014)
Gut permeability problems	Colostrum powder	Preventing gut stability	20 g daily	Marchbank et al. (2011)
Inflammation in colon	Colostrum liquid	Decreasing in symptoms	100 ml daily	(Khan et al., 2002
Acute infectious diarrhea	Colostrum powder	Diarrhea and lower frequency of vomiting	3 g sachet with 50 ml water	(Menchetti et al., 2016; Barakat et al., 2019)

are almost entirely degraded due to their sensitive structure, though sterilization otherwise extends shelf-life for bovine colostrum products and eliminates all viable microorganisms. Sterilization can be tolerated in processing of milk and milk-based products, but this process causes considerably decreased bovine colostrum quality. As a result, typical pasteurization methods can be practical to maintain quality and feeding characteristics of bovine colostrum. Drying methods are also used to preserve therapeutic potential of colostrum immunoglobulins. Freeze drying is the favored dehydration method for heat-sensitive biological samples due to its low processing temperature. This method is useful for minimizing nutrient loss and obtaining a high quality final product. In the context of bovine colostrum, some research indicates that freeze-drying preserves bioactive proteins, enhancing the quality and shelf-life of bovine colostrum as compared to spray drying.

In addition to these processes, some advanced technologies such as nanotechnology and liposomal technology use encapsulation processes to deliver bovine colostrum in a finished product. Various commercial forms of bovine colostrum as end-products are developed with different processes and used for different purposes, each choosing the advantages of a particular processing scheme for the desired end use. The interest in using bovine colostrum in some food products is increasing and it is considered to be a novel functional food.

AUTHOR CONTRIBUTIONS

SK organized the general content of the paper. MerK was responsible for general editing and organizing the authors as well as the contribution for two sections. AA, HD, MelK, BB, BBG, MA, AB, HIT, ME, and GE contributed one section of the paper. RMD, BMH, and SAF were responsible for the editing and organizing the paper. All authors contributed to the article and approved the submitted version.

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Resveratrol Relaxes Human Gastric Smooth Muscles Through High Conductance Calcium-Activated Potassium Channel in a Nitric Oxide-independent Manner

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Resveratrol, as a polyphenolic compound that can be isolated from plants, and also a component of red wine has broad beneficial pharmacological properties. The aim was to investigate the role of nitric oxide and potassium channels in resveratrol-induced relaxation of human gastric smooth muscle. Gastric tissues were obtained from patients who underwent sleeve gastrectomy for severe obesity (n = 10 aged 21–48; BMI 48.21 ± 1.14). The mechanical activity from the muscle strips was detected under isometric conditions as the response to increasing concentrations of resveratrol before and after different pharmacological treatments. Resveratrol caused an observable, dose-dependent gastric muscle relaxation. The maximal response caused by the highest concentration of resveratrol was 83.49 ± 2.85% ($p < 0.0001$) of the control. Preincubation with L-NNA, L-NAME, or ODQ did not prevent the resveratrol-induced relaxation. Apamin, glibenclamide, 4AP or tamoxifen, did not inhibit the relaxing effect of resveratrol, as well. In turn, blocking BK_{Ca} by TEA, iberiotoxin, or charybdotoxin resulted in inhibition of resveratrol-induced relaxation (91.08 ± 2.07, $p < 0.05$; 95.60 ± 1.52, $p < 0.01$ and 89.58 ± 1.98, $p < 0.05$, respectively). This study provides the first observation that the relaxant effects of resveratrol in human gastric muscle strips occur directly through BK_{Ca} channels and independently of nitric oxide signaling pathways. Furthermore, there is considerable potential for further extensive clinical studies with resveratrol as an effective new drug or health supplement to treat gastrointestinal dyspepsia and other gastric hypermotility disorders.

Keywords: resveratrol, smooth muscles, gastric motility, potassium channels, nitric oxide

INTRODUCTION

Resveratrol, as a naturally occurring bioactive molecule, has become a popular subject of scientific interest due to its potential benefit functions. Resveratrol belongs to a large group of natural, plant-derived polyphenols and phytoestrogens and can be found e.g., in grapes, berries, or red wine (Berk et al., 2019; Repossi et al., 2020). Numerous studies report its: anti-proliferative, anti-oxidative, anti-inflammatory, cytoprotective, anti-microbial, anti-dyslipidemia, or anti-diabetic properties (Charytoniuk et al., 2018; Repossi et al., 2020). Moreover, due to multiple pharmacological effects and promising results of preclinical studies, in many clinical trials over the past few years, oral supplementation with combinations of, inter alia, resveratrol has been introduced (Drygalski et al., 2018).

According to previous research, the clinical use of resveratrol is considerably limited due to the low oral bioavailability caused by the short biological half-life, poor water solubility, and rapid metabolism (Palle and Neerati, 2017; Peng et al., 2018). Therefore, studies are conducted to achieve an effective serum concentration of this polyphenol to reach many of the proposed sites of action outside the gastrointestinal (GI) tract after an oral dose of 25 mg to even 5 g (Peng et al., 2018; Wang and Sang, 2018). Moreover, efforts have been made to modify resveratrol for improved bioavailability and reduced toxicity (Smoliga and Blanchard, 2014). The process of micronization was used to enhance resveratrol absorption across the gastrointestinal tract (Vesely et al., 2021). One of the most interesting aspects of its future development as a promising drug is that resveratrol does not appear to have side effects at short-term dosages, and no major side effects have been found in long-term clinical trials. At doses of 2.5 g or more per day, side effects such as nausea, vomiting, and diarrhea may occur (Salehi et al., 2018; Shaito et al., 2020). However, thus far little attention has been given to the relaxing effect of the human GI smooth muscle caused by resveratrol.

GI motility is a particularly important and complex physiological function of the digestive tract, regulated by many factors. The main factor responsible for GI symptoms is gastric mobility dysfunction. Sex hormones, estrogens, in particular, are known to cause GI motility disorder and contribute to irritable bowel syndrome (Zhang et al., 2014). Resveratrol is structurally and functionally similar to estrogens and belongs to the group of phytoestrogens. Recent data indicate that resveratrol may inhibit the contractility of the GI tract in rats and guinea pigs (Zhang et al., 2014). Despite a large number of studies on the beneficial effects of resveratrol on smooth muscles, the underlying mechanisms are not fully understood. The relaxation of GI smooth muscles is controlled by non-adrenergic non-cholinergic (NANC) signaling regulated by neurons of the myenteric plexus located between the circular and longitudinal muscle layers (Van Geldre and Lefebvre, 2004). One of the main NANC mediators is nitric oxide (NO), a gaseous neurotransmitter synthesized from L-arginine by nitric oxide synthase (NOS) in response to neuronal stimulation (Bult et al., 1990). NO increases the cellular cGMP level, which leads to the K^+-channel activation: high conductance Ca^{2+}-dependent (BK_{Ca}) and small conductance apamin-sensitive (SK_{Ca}) (Matsuda and Miller, 2009). Furthermore, direct activation of cGMP-independent K^+-channels, as well as inhibition L-type Ca^{2+}-channels, is mediated by the NO pathway (Matsuda and Miller, 2009). All these molecular mechanisms lead to hyperpolarization of the cell membrane resulting in smooth muscle relaxation.

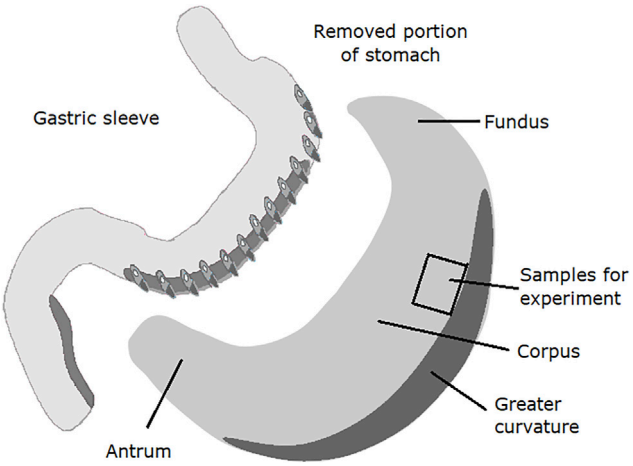

FIGURE 1 | Diagram of anatomical areas of the stomach after gastrectomy using the vertical sleeve method. The place of sampling for the conducted experiments was marked.

Resveratrol can modify nitric oxide (NO) levels by its action on both endothelial NOS and cytokine-inducible NOS (Kline and Karpinski, 2015). Moreover, resveratrol exerts both indirect and direct vasodilator effects on blood vessels by NO-mediated and non-NO-mediated mechanisms (Choi et al., 2016). Furthermore, only a few studies of the effects of resveratrol in the gastrointestinal smooth muscle have been conducted and its molecular mechanism of action remains unclear.

Our study aimed to investigate the role of nitric oxide and potassium channels in resveratrol-induced relaxation of human gastric smooth muscle. Furthermore, the results of our preclinical research may become the basis for further extensive clinical studies with resveratrol in the treatment of gastrointestinal dyspepsia and other gastric hypermotility disorders.

MATERIALS AND METHODS

Sample Processing for Isometric Contraction

The study was conducted under the Helsinki Declaration principles, the International Conference on Harmonization Guideline for Good Clinical Practice, the laws and regulations of Poland, and with the approval from the local Ethical Committee (No. R-I-002/304/2018). Human gastric tissues were obtained from patients who underwent sleeve gastrectomy for morbid obesity (n = 10 aged 21–48; BMI 48.21 ± 1.14). The collection of the tissues did not interfere

with the surgical procedure. Samples were taken from the upper half of the stomach with larger curvature removed during the surgical procedure (**Figure 1**) (Hady et al., 2012). All patients were carefully informed about the aim and nature of the study before surgery and signed written consent.

All surgeries were performed under general anesthesia performed by the same team of anesthesiologists. Combined general anesthesia was induced by propofol (1.0–1.5 mg per kg body mass) and opioid analgesic fentanyl (1.0–1.5 μg per kg body mass). A non-depolarizing neuromuscular blocking agent cisatrocurium (0.1–0.2 mg per kg body mass) was also administered at that stage. Maintenance of general anesthesia was achieved with the volatile method with sevoflurane administered in repetitive doses. Additional doses of cisatrocurium and opioids were also administered depending on the patient's needs and metabolism.

After removal, specimens were immediately cooled on ice-cold Tyrode's buffer, bubbled with carbogen (95% O_2 +5% CO_2), and transported to the laboratory, and then treated as previously described (Pedzińska-Betiuk et al., 2011). Subsequently, the muscle layer was dissected from the gastric wall and cut in the direction of the longitudinal muscles into 10 mm × 3 mm × 1.5 mm strips. The tissues were attached to an isometric force transducer and placed in 20 ml tissue bath chambers. The bath temperature was kept constantly at 37°C and the carbogen was continuously bubbled. The preparations were allowed to equilibrate for 1 h. During this period the passive tension was adjusted to 2 mN and the organ bath solution was exchanged every 20 min. Before each experiment, strips were activated by 80 mmol/L K^+. Only strips showing a stable response to potassium were used in the experiments (Modzelewska et al., 2019; Modzelewska et al., 2017).

Experimental Protocol

After the equilibration period, contractile activity was stimulated using carbachol (10^{-6} mol/L) and taken as control after reaching its plateau. Then, resveratrol was added cumulatively to the organ chambers in the range from 10^{-7} to 10^{-4} mol/L every 10 min. To eliminate the effects of the resveratrol solvent, the influence of ethanol alone at the same concentration on gastric strips was investigated. The solvent did not influence the tone of the tissues, the responses to the relaxant agent were reproducible in the control strips unless otherwise stated. Furthermore, NOS synthase blockers (L-NNA, L-NAME—both 10^{-5} mol/L) and a soluble guanylate cyclase blocker (ODQ—10^{-6} mol/L) were used to establish the role of NOS in the relaxing effect of resveratrol. Additionally, concentration-response curves for resveratrol were constructed in the absence and presence of various potassium channels blockers to investigate their involvement. A non-selective K^+ channel blocker—tetraethylammonium chloride (TEA—10^{-3} mol/L), a selective inhibitor of BK_{Ca}—10^{-7} mol/L iberiotoxin (IbTX), an inhibitor of BK_{Ca}, and intermediate (IK_{Ca}) conductance calcium-activated potassium channels (K_{Ca}), and slowly inactivating voltage-gated K_{Ca}—10^{-7} mol/L charybdotoxin (ChTX), an SK_{Ca} blocker—10^{-6} mol/L apamin, a K_V channel blocker—10^{-3} mol/L 4-Aminopiridine (4AP) or a K_{ATP} channel blocker—10^{-6} mol/L glibenclamide, and a selective estrogen response modifier, and protein kinase C inhibitor—10^{-6} mol/L tamoxifen were added 20 min before the addition of resveratrol. Whenever possible, experiments were conducted with tissues obtained from the same patient and were tested in parallel. In a separate series of experiments, controls were performed under comparable conditions of the experiment and at the same time.

Contraction Measurements

Data acquisition was performed by an isometric force transducer (BIO-SYS-TECH, Bialystok, Poland). The following parameters such as the area under the curve (AUC), average baseline muscle tone, and relative change in muscle contraction were evaluated with the DASYLab software (version 9.0; Laboratory Data Acquisition System, SuperLogics, Waltham, MA, United States). The results were presented as the stress–stretch ratio. All outcomes from two to four strips from each sample were averaged at the 10-min interval for each dose of the substance used. The AUC revealed the contractile activity of examined sample responses before and after the administration of the given drug. (Gagnon and Peterson, 1998; Modzelewska et al., 2003). The values of the AUC were assessed by calculating the integral of the suitable 10-min interval of the curve. Concentration-response curves were fitted to the logistic equation using nonlinear regression Y=Bottom+(Top-Bottom)/(1 + 10^((LogEC50-X)*HillSlope)) (PRISM 6.0, GraphPad Software Inc., San Diego, CA, United States). The maximum relaxing response (E_{max}) was presented as a percentage of the values obtained just before the addition of the test substance. Subsequently, the concentrations of a compound where 50% of its maximal effect is observed were expressed as -log EC_{50}. The values were demonstrated as the standard error of the mean (±SEM) of sample data performed on strips from various patients or as the percentage change [(effect size—baseline contractile response)/baseline contractile response × 100]. Results from pairs of responses were averaged.

Chemicals

Resveratrol (5-[(1*E*)-2-(4-Hydroxyphenyl)ethenyl]-1,3,benzenediol) was purchased from abcr GmbH (Karlsruhe, Germany). Carbamylcholine chloride ((2-Hydroxyethyl)trimethylammonium chloride carbamate; carbachol), N^G-Methyl-L-Arg (Nω-Nitro-L-arginine methyl ester hydrochloride, L-NAME), N5-(Nitroamidino)-L-2,5-diaminopentanoic acid, N^G-NO_2-L-Arg ($N_ω$-Nitro-L-arginine, L-NNA), 1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one (ODQ), iberiotoxin (IbTX), charybdotoxin (ChTX), apamin, glibenclamide, N,N,N,N-Tetraethylammonium chloride (TEA), 4-Aminopiridine (4AP) and tamoxifen were purchased from the Sigma Chemical Company, were purchased from Sigma (St. Louis, MO).

Resveratrol was dissolved in 70% ethanol so that the final concentration of ethanol was never >0.1%, which did not affect basal contraction. The working solutions were prepared fresh on the day of the experiment by diluting the stock solution.

Stock solutions of carbachol, L-NNA, L-NAME, apamin, IbTX, ChTX, TEA, 4AP, and tamoxifen were prepared with bidistilled water,

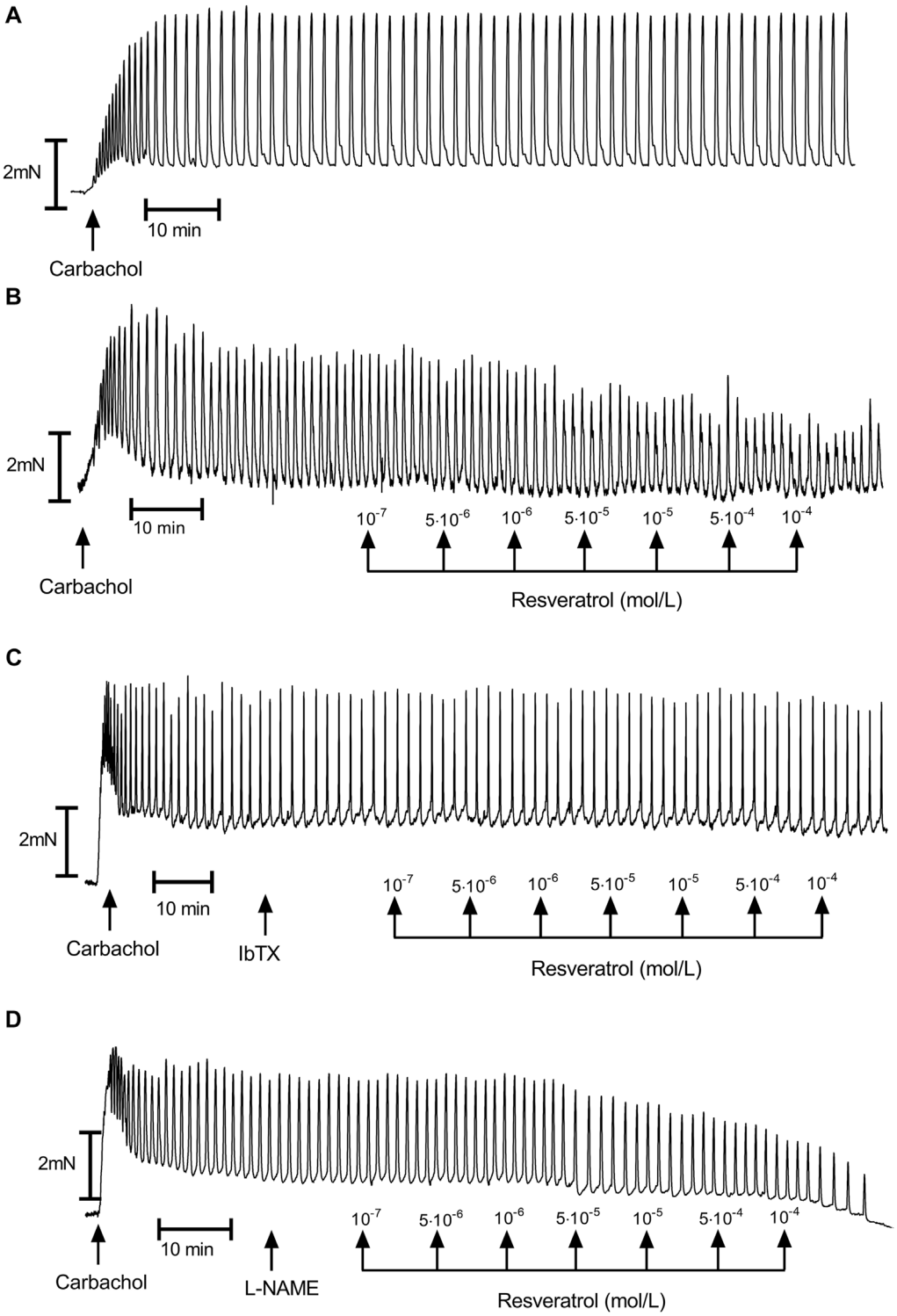

FIGURE 2 | A representative tracings of carbachol-induced gastric muscle contractions. **(A)**—control; **(B)**—resveratrol (range 10^{-7}–10^{-4} mol/L); **(C)**—resveratrol (range 10^{-7}–10^{-4} mol/L) after preincubation with iberiotoxin (10^{-7} mol/L); **(D)**—resveratrol (range 10^{-7}–10^{-4} mol/L) after preincubation with L-NAME (10^{-6} mol/L).

and glibenclamide and ODQ were dissolved in dimethyl sulphoxide (DMSO). The given concentrations were the calculated final concentrations in the organ bath solution. All reagents were added directly to the bath fluid containing a Tyrode's solution composed of (mmol/L): NaCl 139.6; KCl 2.68; $MgCl_2$ 1.05; NaH_2PO_4 1.33; $CaCl_2$ 1.80; $NaHCO_3$ 25.0; and glucose 5.55.

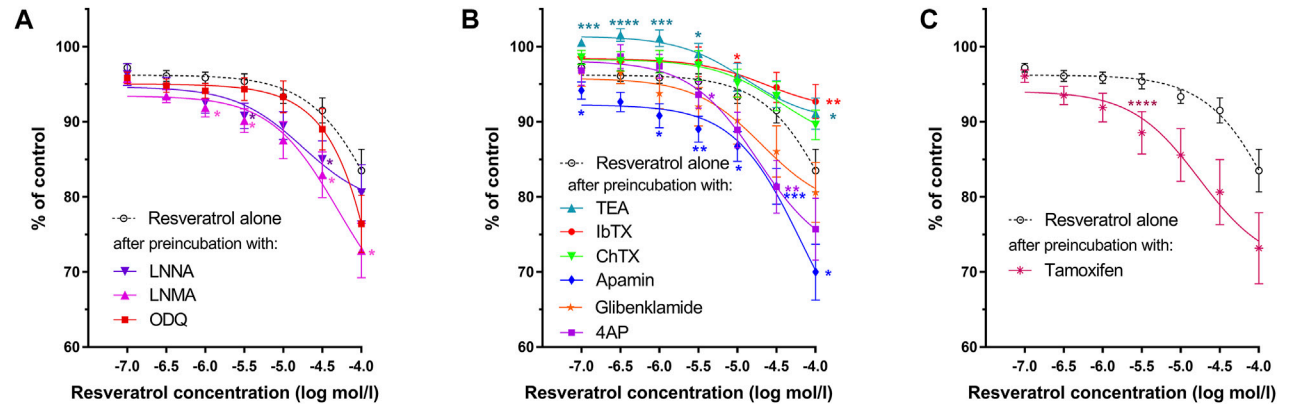

FIGURE 3 | Effects of resveratrol on the gastric muscle samples as measured by AUC after preincubation with **(A)**—L-NNA, L-NAME, ODQ; **(B)**—TEA, IbTX, ChTX, apamin glibenclamide or 4AP; **(C)**—tamoxifen. Each point represents the mean ± SEM of AUC values obtained from individual strips (n = 10) from different patients. AUC of carbachol-induced contractions of each gastric strip over a 10-min interval before the addition of the antagonist was treated as control. Significance at $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ as compared to the AUC of resveratrol alone.

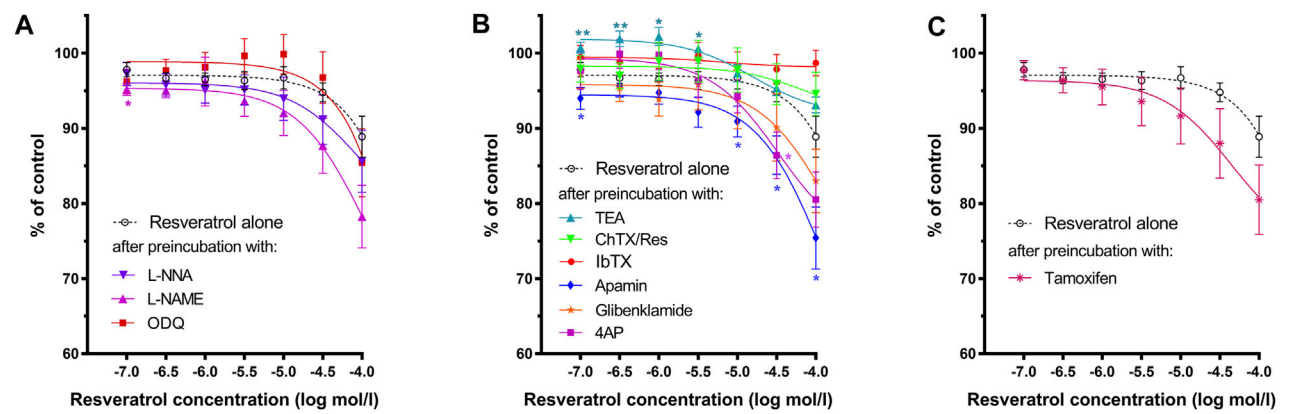

FIGURE 4 | Effects of resveratrol on the basal tension of gastric muscle samples after preincubation with **(A)**—L-NNA, L-NAME, ODQ; **(B)**—TEA, IbTX, ChTX, apamin glibenclamide or 4AP; **(C)**—tamoxifen. Each point represents the mean ± SEM of basal tension values obtained from individual strips (n = 10) from different patients. Basal tension of carbachol-induced contractions of each gastric strip over a 10-min interval before the addition of the antagonist was treated as control. Significance at $^{*}p < 0.05$; $^{**}p < 0.01$ as compared to the basal tension of resveratrol alone.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, United States). The D'Agostino-Pearson test was used to determine the normal distribution of the investigated variables, and then the compliance with the Gaussian distribution was checked. For comparing values of the two following measurements, the one-way ANOVA or the Kruskal-Wallis test was used, where appropriate. Statistically significant differences between means were determined by Tukey's *post-hoc* or a nonparametric Mann-Whitney *U* test, where appropriate. A probability value of less than 0.05 was regarded as significant.

RESULTS

Carbachol (10^{-6} mol/L) promoted noticeable and long-duration contractions in muscle strips isolated from the upper half of the human stomach (**Figures 1**, **2**). Typical tracings show the response of gastric smooth muscle strips to cumulatively applied resveratrol (**Figure 2**).

Resveratrol induced a definite, dose-dependent relaxation of the gastric strips. (**Figures 2B**, **3**, **4**), substantial—at 10^{-7} mol/L and maximal—at 10^{-4} mol/L. The maximal response caused by 10^{-4} mol/L resveratrol was extremely significant—83.49 ± 2.85% (n = 10; p = 0.0003) of the contractions of the strips before resveratrol administration (**Figure 3** and **Table 1** and **Supplementary Table S1**). Resveratrol dose-dependently reduced baseline tension at all concentrations. The reduction was considerably lowered the resting tension only in 5×10^{-6} and 10^{-4} mol/L (**Figure 4** and **Table 1** and **Supplementary Table S2**).

As shown in **Figure 3A** and **Table 1**, both NOS (L-NNA, L-NAME) blockers and a guanylate cyclase blocker (ODQ) did not affect the course of the relaxing response of the samples

TABLE 1 | Log EC_{50} and E_{max} for resveratrol on carbachol-induced contractility of the human gastric muscles. The values are mean ± SD of $n = 10$ individual gastric strips from different patients. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ versus resveratrol alone; $^{¥}p < 0.05$ versus preincubation with IbTX.

	$logEC_{50}$	*p*	E_{max}	*p*
Resveratrol alone	−3.75 ± 0.19		83.49 ± 2.85	
Resveratrol after preincubation with				
L-NNA	−4.75 ± 0.11***	$p < 0.001$	80.55 ± 3.75	0.610
L-NAME	−4.29 ± 0.09*	0.020	72.84 ± 3.64	0.040
ODQ	−2.46 ± 2.75	0.640	76.40 ± 3.82	0.070
TEA	−4.90 ± 0.29****	$p < 0.0001$	91.08 ± 2.07*	$p < 0.05$
IbTX	−3.72 ± 2.97	0.980	95.60 ± 1.52**	$p < 0.010$
ChTX	−4.44 ± 0.48*	0.011	89.58 ± 1.98¥	0.040
Apamin	−4.18 ± 0.09	0.056	69.96 ± 3.72*	0.010
Glibenclamide	−4.69 ± 0.13***	$p < 0.001$	80.59 ± 3.97	0.490
4AP	−4,74 ± 0.25***	$p < 0.001$	75.68 ± 4.13	$p = 0.09$
Tamoxifen	−4.76 ± 0.10***	$p < 0.001$	73.17 ± 4.74	0.138

induced by resveratrol ($n = 10$). Also, when the basal tension changes were observed, no clear effect of preincubation with the above blockers was observed. (**Figures 2D**, **4A**). Yet, we observed a statistically significant shift to the left of the concentration-response curve for resveratrol after preincubation with L-NNA ($p < 0.001$) or L-NAME ($p < 0.05$) compared with the experiments without NOS blockers (**Table 1**). These results indicate that resveratrol-induced relaxation does not involve the activation of the nitric oxide pathway.

Conversely, ChTX ($n = 10$) tended to inhibit resveratrol-induced relaxation. Preincubation with IbTX inhibited the relaxing effect of resveratrol, which was statistically significant at concentrations higher than 5×10^{-5} mol/L (**Figures 2C**, **3**, **4**). A non-selective K^+ channel blocker—TEA ($n = 10$), as well as selective BK_{Ca} channel blockers (IbTX and ChTX), reduced the relaxant effects of resveratrol, which was extremely significant in low resveratrol concentrations and statistically significant in the highest concentration of the AUC values, and between basal tension values below 10^{-5} mol/L (**Figure 3B**; **Table 1** and **Supplementary Table S1**).

Apamin ($n = 10$) enhanced the relaxing effect of the concentration-response curve of resveratrol. The difference between AUC values was substantial at resveratrol concentrations above 10^{-6} mol/L (extremely at 5×10^{-4} mol/L) and between basal tension values above 5×10^{-5} mol/L (**Figures 3B**, **4B**; **Table 1**, **Supplementary Table S1, S2**). Preincubation with glibenclamide (n = 10) did not significantly alter the relaxing effect of resveratrol on AUC and basal tension. However, there was an extremely significant left shift from the concentration-response curve for resveratrol ($p < 0.001$) (**Figures 3B**, **4B** and **Table 1**, **Supplementary Table S1, S2**).

Blocking K_v -dependent K^+ channels by 4AP ($n = 10$) enhanced the relaxant effect of resveratrol, statistically significant in almost all resveratrol concentrations (**Figure 3B** and **Table 1**, and **Supplementary Table S1**). Preincubation with 4AP lowered the basal tension compared with the experiments without 4AP (**Figure 4B** and **Table 1**, and **Supplementary Table S2**) The effect was statistically substantial in the lowest and higher than 5×10^{-5} mol/L concentrations.

Incubation of gastric smooth muscle strips with tamoxifen enhanced resveratrol-induced relaxation, extremely substantially at 5×10^{-5} mol/L (AUC) without significant influence on their basal tension (**Figures 3C**, **4C**, **Supplementary Table S1**). For tamoxifen, also a statistically substantial shift to the left of the concentration-response curve for resveratrol was observed ($p < 0.001$) (**Table 1**).

DISCUSSION

Resveratrol has been shown to relax various types of smooth muscle, including arteries (Naderali et al., 2001; Shen et al., 2013; Choi et al., 2016; Breuss et al., 2019), umbilical vein (Hassanpour et al., 2021), uterus (Wu et al., 2015), gallbladder (Kline and Karpinski, 2015), corpus cavernosum (Soner et al., 2010) and GI tract (Zhang et al., 2014; Parlar and Arslan, 2019; Modzelewska et al., 2021). In previous studies, doses were used in a fairly wide range, and due to the low oral bioavailability of resveratrol, an orally administered nutraceutical operated almost exclusively in the gastrointestinal tract (Palle and Neerati, 2017; Peng et al., 2018). Considering the above, we decided to examine the influence of this polyphenol on the human gastric muscles contractions *in vitro*.

We found resveratrol concentration-dependently decreased human gastric muscle contractions in all concentrations used. Our finding is consistent with data reported for the influence of flavonoids on GI muscles (Zhang et al., 2014; Modzelewska et al., 2021).

Due to the structural similarity between resveratrol and synthetic estrogen diethylstilbestrol (Zhang et al., 2014), we investigated whether resveratrol might exhibit an estrogenic effect on GI motility. However, the results of the present study show that this is unlikely, as the relaxing effect of resveratrol was not inhibited by tamoxifen. On the contrary, tamoxifen tended to enhance resveratrol-induced relaxation. A statistically significant left shift in the concentration-response curve can be attributed to the fact that resveratrol acts as an agonist at the estrogen GPER-1 receptor (Levenson et al., 2003). Hence, in the control group, some resveratrol molecules are bound to these receptors. When incubated with tamoxifen, the resveratrol binding may have

become blocked. Therefore, compared to control, a greater amount of resveratrol particles was available to interact with BK_{Ca} channels, thus enhancing relaxation.

The ability of NO to relax smooth muscles is well established (Modzelewska et al., 2019, Modzelewska et al., 2021; Idrizaj et al., 2021). One of the mechanisms of NO smooth muscle relaxation is the activation of soluble guanylyl cyclase (sGC) (Friebe et al., 2018), which is expressed in a variety of cell types and signals through cGMP on to cGMP-dependent protein kinase (PKG), phosphodiesterases or, possibly, on to cGMP-regulated channels (Friebe and Koesling, 2003). Previous research has demonstrated that resveratrol-induced smooth muscle relaxation was primarily related to NO and potassium channels. (Choi et al., 2016). The NO pathway *via* cGMP-dependent or independent mechanisms has been reported to be involved in the regulation of the muscle tone of the GI relaxation response. (Rocha et al., 2014). Therefore, the influence of the NO pathway on the mechanism of action of polyphenols in the GI is not clearly defined. Tsai et al. demonstrated that the resveratrol-induced relaxation of the guinea pig fundus was inhibited by L-NNA, indicating that this process is mediated by both the NOS of the neuron or the smooth muscle of the fundus (Tsai et al., 2018). However, Zhang et al. presented that resveratrol relaxes GI smooth muscle partially by nitrergic pathway (Zhang et al., 2014). In turn, Amira et al. showed the inhibitory effect of flavonoids on the gastric tone in mouse isolated stomach strips was not significantly reduced in the presence of L-NAME, suggesting that it is not related to NO production (Amira et al., 2008).

In the present study, we demonstrated that resveratrol-induced relaxation was not inhibited by the NOS inhibitor L-NNA or L-NAME, and the sGC inhibitor ODQ, as well. The -log EC_{50} for the effect of resveratrol after preincubation with L-NNA ($p < 0.001$) or L-NAME ($p < 0.05$) showed a statistically significant shift to the left versus a curve without NOS blockers. In our experiment, blocking soluble guanylyl cyclase (sGC) by ODQ virtually did not change the relaxing effect of resveratrol. Therefore, it is possible that blocking the activation of the NO pathway can enhance the relaxant effects of resveratrol of the human gastric smooth muscle and may indicate the complexity of the process of calcium release from the sarcoplasmic reticulum. It can be hypothesized that this process, which may be mediated by resveratrol or NO, is not synergistic but competitive. This could further support the leading conclusion of our research that the main mechanism of resveratrol triggering the relaxation of gastric smooth muscle is the activation of Ca^{2+}-dependent K^+ channels. This preliminary hypothesis requires more in-depth analysis and experimental confirmation. Presented data is in line with our previous results for quercetin. There, too, no effect of blocking the NO pathway on gastric muscle relaxation was observed. (Modzelewska et al., 2021). Thus, our findings indicate that resveratrol can cause the human gastric muscles to relax independently of the NO pathway. On the other hand, different contractile stimulants and species may influence the difference in resveratrol-induced relaxation mechanisms between rats, guinea pigs, and human GI smooth muscle. Thus, more research is needed to elucidate the difference between the different species.

Since neither L-NNA, L-NAME nor ODQ inhibited resveratrol-induced relaxation of human gastric strips, we investigated the effect of blocking various types of K^+ on this effect in subsequent experiments. Activation of K^+ channels of the cell membrane causes its hyperpolarization and additionally suppresses the influx of Ca^{2+} into the cell, causing relaxation of smooth muscles. Apamin, SK_{Ca} channels blocker as well as glibenclamide (K_{ATP} channels blocker) did not inhibit the relaxing effect of resveratrol. However, there was a statistically significant left shift from the -logEC_{50} curve for resveratrol ($p < 0.001$) after preincubation with glibenclamide. This raises questions as to the mechanism of such enhancement of the relaxing effect of resveratrol. When we block K_{ATP} channels, in addition to inhibiting the outflow of K^+ ions from the cell, the membrane potential also changes (Brayden, 2002). Under their regulation, activation of voltage-gated calcium channels is possible, calcium influx, and possibly additionally activation of Ca^{2+}-dependent K^+ channels. The verification of this hypothesis requires further research. Instead, blocking BK_{Ca} by a specific blocker of these channels IbTX resulted in complete inhibition of resveratrol-induced relaxation. ChTX also inhibited the effect of resveratrol, but this effect was weaker than that of IbTX. This is probably because ChTX is a non-specific BK_{Ca} channel blocker acting simultaneously on the IK_{Ca} channels and slowly inactivating voltage-gated K^+ channels (Kv1.3) (Giangiacomo et al., 1993). Tan et al., 2020 demonstrated that resveratrol targets multiple signaling pathways to exert a vasorelaxant effect in a rat aortic ring model specifically can suppress not only extracellular calcium influx but also an intracellular release of calcium from the sarcoplasmic reticulum within vascular smooth muscle cells (Tan et al., 2020). Then it should be considered, what if resveratrol works by inhibiting Ca^{2+} channels? The consequence of Ca^{2+} channel inhibition is a subsequent decrease in Ca^{2+} concentration inside the cell, followed by muscle relaxation. However, at the same time, Ca^{2+}-dependent K^+ channels remain closed, because the factor that causes them to open is an increase in the Ca^{2+} concentration inside the cell (Borowiec et al., 2014). Therefore, blocking them by iberiotoxin, charybdotoxin or TEA should not affect their activity, and thus the strength of muscle contraction. In our experiment, we demonstrated that blocking these Ca^{2+}-dependent K^+ channels abolished the relaxing effects of resveratrol. Hence, we can conclude that resveratrol acts either directly on BK_{Ca} channels to open them or acts locally to release small amounts of Ca^{2+} from the sarcoplasmic reticulum which is in contrast to the Tan et al., 2020 findings. BK_{Ca} channels are considered channel complexes formed by an ion-conducting α-subunit and regulatory β (β_{1-4})- or γ (γ_{1-4})-subunits (Sancho and Kyle, 2021). They have been reported to be modulated by depolarization, calcium, stretch-triggered, independently and even if the Ca^{2+} channel is inhibited, BK_{Ca} may still be activated (Qi et al., 2005; Wang et al., 2010; Xin et al., 2018). However, little is known about the mechanisms underlying the activation and termination of Ca^{2+} sparks in muscle (Fill and Copello, 2002; Vesely et al., 2021). Moreover, the detailed molecular mechanism that activates BK_{Ca} channels by membrane stretch remains unclear, nevertheless, the modulating role is attributed to the β_1-subunit of the BK_{Ca} channel (Xin et al., 2018). Therefore, in our research, we used ChTX and the highly selective IbTX, which act as pore blockers, occluding conduction pathways of the α-subunit, and are useful experimental tools, mainly due to their poor reversibility and exclusion of the influence of other units. Additionally, a quaternary amine, such as TEA blocks BK_{Ca} channels through

either the internal or external side of the membrane, implying a complex mechanism of action in a voltage-dependent manner (Contreras et al., 2013; Sancho and Kyle, 2021). Hence, our results support the idea that resveratrol induces the relaxation of the smooth muscles of the human stomach through the activation of BK_{Ca} channels.

Few studies have been published explaining resveratrol-induced smooth muscle relaxation, and those that exist propose different pathways of action of this polyphenol. Moreover, they are mainly animal studies. Tsai et al. have shown that resveratrol-induced relaxation of the guinea pig fundus occurs through the NO pathway and K_{ATP} channels (Tsai et al., 2018). In turn, Zhang et al. reached a different conclusion by studying rat gastrointestinal smooth muscle. Their results indicate that resveratrol relaxes rat's gastrointestinal smooth muscle *via* α-adrenergic receptors, NO and cyclic adenosine monophosphate pathways, K_{ATP} channels, and inhibition of L-type Ca^{2+}channels (Zhang et al., 2012). Several papers also describe the relaxant effects of this polyphenol on rat arteries. Gojkovic-Bukarica et al. showed that this process involves activation voltage-gated K^+ channels (Novakovic et al., 2006; Gojkovic-Bukarica et al., 2008). The same mechanism is also indicated by the same authors for the relaxation of the rat renal artery (Gojkovic-Bukarica et al., 2019). In turn, Dalaklioglu and Ozbey have shown that 4AP did not significantly alter relaxant responses of rat corpus cavernosum strips to resveratrol (Dalaklioglu and Ozbey, 2014). In our experiments, concentration-response relationships of resveratrol and 4AP, a non-specific blocker of K_V channels, have shown that K_V channels do not participate in the relaxing effect of resveratrol on human gastric smooth muscle. Membrane depolarization activates K_V channels, and, in general, they participate in the negative feedback regulation of muscle contraction along with BK_{Ca} channels. Consistent with this negative-feedback role, block of K_V channels potentiates contraction induced by vasoconstrictors (Jackson, 2017). And since, rather than contraction, an enhancement of resveratrol-induced relaxation is observed, it could be suggested that the process is competitive rather than synergistic. On the other hand, Chen et al., also examining the rat aorta, indicated a different, by NO-mediated mechanism of relaxation (Chen and Pace-Asciak, 1996). Regarding the *in vitro* studies of human smooth muscles, these concerned the evaluation of the relaxing effect of resveratrol on the muscles of the gallbladder. The results of the above research show that inhibition of contractions is associated with NO, K_{ATP} channels, and BK_{Ca} channel pathways (Tsai et al., 2017). While all authors agree that resveratrol has a relaxant effect on smooth muscles, it can be noted that the proposed mechanisms describing this process differ considerably. Importantly, the muscle relaxant effect of resveratrol, despite its low bioavailability, may partly explain its influence on the oral glucose tolerance test in diabetic studies (Jakubczyk et al., 2020). The reduction of GI tract motility lowers the stomach emptying rate what affects the pharmacokinetics of glucose or digested meals preventing postprandial hyperglycemia. This opens the way for further research of this compound both at the level of pharmacokinetics and its molecular effects in tissues.

Dietary resveratrol and/or its metabolites, as they pass along the GI tract, possess a wide spectrum of valuable local physiological and pharmacological effects. On a systemic level, its absorption may exhibit a wide range of beneficial properties. The impact of regional differences in anatomy and physiology across the GI tract on drug absorption is significant and for some classes of active pharmaceutical ingredients, such as orally administered polyphenols, it is a challenge to understand the nature of these barriers. Accordingly, the beneficial effects of these compounds, including resveratrol, may occur *in situ*. Moreover, in the results of clinical trials to date, there have been no reports of the relaxing properties of resveratrol in the gastrointestinal tract. (Salehi et al., 2018; Singh et al., 2019; Shaito et al., 2020). This indicates that it may be beneficial to conduct a study focusing on the relaxant properties of resveratrol in gastrointestinal disorders. Therefore, it is worth researching resveratrol as well as other nutraceuticals that increase gastric muscle relaxation as a potential drug to functional dyspepsia.

CONCLUSION

In conclusion, this study provides the first observation that the relaxant effects of resveratrol in human gastric muscle strips occur directly through BK_{Ca} channels and independently of NO pathways. Furthermore, there is considerable potential for further extensive clinical studies with resveratrol as an effective new drug or health supplement to treat gastrointestinal dyspepsia and other gastric hypermotility disorders.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Medical University of Bialystok (No. R-I-002/304/2018). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BM: Conceptualization, Methodology, formal analysis and investigation, writing—original draft preparation, review and editing, funding acquisition, supervision; KD: Conceptualization, formal analysis, writing—review, funding acquisition; HH: Methodology, resources, writing—review; AK: Formal analysis, writing original draft preparation; AC: Formal analysis and investigation, writing—review; KK; Formal analysis, writing original draft preparation; PW: Methodology, Resources; TK: Conceptualization, methodology, formal analysis, and investigation; writing—review and editing, supervision. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.823887/full#supplementary-material

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Construction of the Enterococcal Strain Expressing Immunogenic Fragment of SARS-Cov-2 Virus

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Contemporary SARS-Cov-2 pandemic, besides its dramatic global influence on the human race including health care systems, economies, and political decisions, opened a window for the global experiment with human vaccination employing novel injectable vaccines providing predominantly specific IgG response with little knowledge of their impact on the mucosal immunity. However, it is widely accepted that protection against the pathogens at the gates of the infection - on mucosal surfaces—predominantly rely on an IgA response. Some genetically modified bacteria, including probiotics, represent attractive vehicles for oral or nasal mucosal delivery of therapeutic molecules. Probiotic-based vaccines for mucous membranes are easy to produce in large quantities; they have low cost, provide quite a long T-cell memory, and gut IgA response to oral vaccines is highly synchronized and strongly oligoclonal. Here we present a study demonstrating construction of the novel SARS-Cov-2 vaccine candidate employing the gene fragment of S1 SARS-Cov-2 gene. This DNA fragment was inserted in frame into major pili protein gene with d2 domain of enterococcal operon encoding for pili. The DNA sequencing proved the presence of the insert in enterococcal genome. RNA transcription, immunoprecipitation, and immune electron microscopy with human sera obtained from the SARS-Cov-2 patients demonstrated expression of SARS-Cov-2 antigens in bacteria. Taken together the data obtained allowed considering this genetically modified probiotic strain as an interesting candidate for vaccine against SARS-Cov-2.

Keywords: probiotic, enterococcus, probiotic-based vaccines, SARS-CoV-2, immune response, S protein

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INTRODUCTION

The onset of the SARS-Cov-2 pandemic required the urgent preventive measures to limit the spread of the virus has accelerated the development of antiviral vaccines. All COVID-19 vaccines which are currently approved or authorized in the United States (Pfizer-BioNTech/Comirnaty, Moderna, and Janssen [Johnson and Johnson]), China, European Union (Astra Zeneca) or Russian Federation (Sputnik V) are effective against COVID-19, including severe disease, hospitalization, and death. Present data suggest lower effectiveness of the present vaccines against confirmed infection and symptomatic disease caused by the Beta, Gamma, and Delta variants compared with the ancestral strain and Alpha variant (Agrawal et al., 2021; Björk et al., 2021; Gushchin et al., 2021; Harder, et al., 2021; Kow and Hasan, 2021; Shapiro et al., 2021; Yin et al., 2021). The majority of the vaccines on the

market indifferently on vaccine making approach, rely on the needle injection of the vaccine hoping for the immune recognition of the viral antigens and for establishment of the cellular and adaptive immune responses to the pathogen in case of its appearance in the organism.

However, such approaches produce a weak immune response on the mucous membranes at the gate of the infection which is oral or gut mucosa allowing the virus to enter the organism. This makes it possible to spread the disease through a fully vaccinated population and provide the possibility of artificial induction of the appearance of viral variants under the pressure of the targeted immune response inflicted by the vaccination. These and some other problems of contemporary vaccination can be solved by mucosal vaccines against SARS-Cov-2 providing the first line of defense against the pathogen. Here we describe the construction and preliminary study of novel mucosal vaccine candidate with enterococcal probiotic as the vector for viral antigens providing immune recognition of SARS-Cov-2.

MATERIALS AND METHODS

Bacterial Cultures

Enterococcus faecium L3 and *Escherichia coli* strains DH5α and M15 were obtained from the collection of the Institute of Experimental Medicine and used as the recipients for transformation. *E. coli* strains were grown in Luria Bertani (LB) medium (Oxoid, United States) at 37°C with constant shaking. *E. faecium* L3 and its derivatives were grown in Todd Hewitt Broth (THB) (HiMedia, India) at 37°C for 14 h. LB agar (Lennox L agar, Thermo Fisher Scientific) and *Enterococcus* Differential Agar Base (TITG Agar Base) (Himedia, India) without antibiotic and with 10 μg/ml of erythromycin were used as a solid medium for cultivation, bacterial quantification, and identification of *E. faecium* L3 and erythromycin-resistant enterococcal transformants. The bacteria *E. coli* M15 SarsS were cultured on Terrific broth in the presence of ampicillin (100mcg/ml) and kanamycin (25 mcg/ml).

Genetic Engineering and Protein Studies

Cloning of the *sars*S Gene Fragment

A fragment of the *sarsS* gene of 512 bp was chemically synthesized and originally cloned into the vector plasmid DNA pAL2-T (Eurogen, Russia).

A fragment of the gene encoding the S-protein of the SARS-CoV-2 virus was obtained by polymerase chain reaction (PCR) using primers Cov1 и Cov2 with incorporated sites for restriction endonucleases *BamHI* and *SacI* and synthesized fragment of the *sarsS* gene encoding the fragment of S-protein of SARS-CoV-2.

The obtained DNA fragment *sarsS* was cloned using the expression plasmid pQE-30 (Qiagen, Hilden, Germany). Recombinant plasmid DNA pQE-sarsS and an expression strain of *E. coli* M15-SarsS were obtained after cloning of the PCR product.

Purification of Recombinant Protein SarsS

After expression in the recombinant *E. coli* M15-SarsS strain protein SarsS was purified under denaturing conditions. Briefly, the bacteria were cultured on Terrific broth in the presence of ampicillin (100 mcg/ml) and kanamycin (25 mcg/ml) until the late logarithmic growth phase (OD 600 = 0.7 ÷ 0.9). Then, the expression of the recombinant protein was induced by the addition of IPTG and the cells were cultured for another 4.5 h. The cells were harvested by centrifugation and the cell pellet was frozen at -70°C. The thawed precipitate was resuspended in the buffer A (8 M urea, 0.1 M Na2HPO4, 0.1 M NaH2PO4, pH = 8.0) and the cells were lysed completely by gentle vortexing for 1 h at room temperature. After removing a cell debris, the protein was purified from the supernatant by using Ni Sepharose. The protein Cov1S eluted from Ni Sepharose (Qiagen, Hilden, Germany), under denaturing conditions revealed a single 24.5 ± 0.5 KDa band by Coomassie brilliant blue staining after 12% SDS-PAGE. The purified protein was refolded using two-step dialysis against 3 M urea, 0.1 M Na2HPO4/NaOH, pH = 9.2 during 2 h and 0.4 M NaCl, 0.02 M Na2HPO4/NaOH, pH = 9.2 overnight at 6°C without stirring. The resulting protein SarsS was sterilized by filtration using Millipore disposable filters (0.45 microns) and stored at 6°C. The analysis of the pure recombinant protein SarsS by MALDI TOF/TOF (Bruker Daltonics, Germany) confirmed that its sequence corresponds to the part of the SARS-CoV-2 S protein (data not shown).

Production of Recombinant *E. faecium* L3-SARS

Development and Cloning of a Fusion Gene *entF-sarsS*

The fusion gene *entF-sarsS* was generated using recombinant suicidal plasmid pentF-*pspf* that was made earlier for making live probiotic pneumococcal vaccine (Gupalova et al., 2018). Plasmid *pentF-pspf* was obtained by inserting *pspf* sequence between two separate gene fragments of the probiotic *Enterococcus faecium* L3. In order to make the plasmid *entF-sarsS*, *pentF-pspf* was digested with *NdeI* and *EcoRI* which were flanking *pspf* sequence. *pspf* sequence was replaced with the chemically synthesized fragment of the *sarsS* gene of (512 bp) which also carried sites for *Nde*I and *Eco*RI for convenience of subcloning.

Resultant plasmid DNA was transformed into the *E. coli* DH5α with selection of the transformants on LA plates with 500 μg/ml erythromycin. To identify the *E. coli* clones with desired plasmid p*entF-sarsS* DNA primers K1 and K2 corresponding to the *sars*S gene were used. (**Table 1**). Plasmid p*entF-sarsS* was isolated employing Mini-Prep kit (Qiagen, Hilden, Germany) and used for electro transformation of enterococci.

Transformation of Enterococcus With an Integrative Plasmid p*entF-sarsS* by Electroporation

For electroporation, *E. faecium* L3 was grown in 3 ml of Todd-Hewitt medium (THB) (HiMedia, India) overnight at 37°C, inoculated (2%) in 50 ml of THB medium and grown to OD_{600} = 0.3. Resultant culture was cooled on ice, washed three times with cold double distilled water with 10% glycerol by centrifugation 3,500 *g* at 4°C. The cell pellet was suspended in 0.5 ml of 10% sterile glycerol solution, precipitated and

TABLE 1 | List of oligonucleotide primers used.

Primers	Direction 5′-3′	Nucleotide sequence from 5′ to 3′	Purpose
K1	Forward	TTGCATATGGGTTTCCAACCCACT[a]	Gene fragment flanking for making *sarsS*
K2	Reverse	GTAGAATTCGTTGTTACATGTTCA	Gene fragment flanking for making *sarsS*
B1	Forward	TGAGTGAACCACAGCCAGAA	Integration of the *pentF-sarsS* plasmid DNA into the *Enterococcus* in chromosomal DNA
Seq F	Forward	GGACACCACAACCATCGAAG	Sequencing of the PCR product of *pentF-S1*
Cov1	Forward	AAGGATCCATACATATGGGTTTCC	Cloning a gene fragment *sarsS* for protein production
Cov2	Reverse	TGTCGACGGAGCTCGAATT	Cloning a gene fragment *sarsS* for protein production
A1	Forward	GCTCTAGAGCCGATGAGAGCAGCTGGT ATTG	Determining the presence of inserts and a fragment of the *sarsS* gene in *Enterococcus*
D1	Reverse	CAACAGGATCCAAAGCATCGTTGG	Determining the presence of inserts and a fragment of the *sarsS* gene in *Enterococcus*
Dal 1	Forward	TTGAGGCAGACCAGATTGACG	D-alanine-D-alanine ligase
Dal 2	Reverse	TATGACAGCGACTCCGATTCC	D-alanine-D-alanine ligase

[a]*The underlined area in the nucleotide sequences correspond to restriction sites used for cloning.*

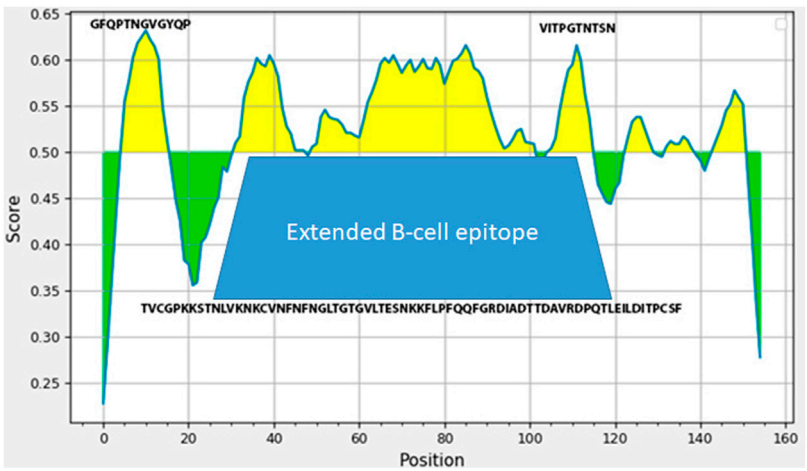

FIGURE 1 | Analysis of SARS-Cov-2 S-protein fragment (amino acids 496-646 in SARS-Cov-2 S protein sequence) employing IDEB (Immune Epitope Database). The amino-acids sequence underneath the trapezoid figure represents large 53 AA immunogenic domain of SARS-Cov-2.

suspended in 0.3 ml of the same solution. A total of 50 µl of cell suspension was added to the electroporation cuvettes with a distance of 1 mm between the electrodes at a voltage of 2100 V.

A total of 300 ng of the integrative plasmid *pentF-sarsS* was added to 50 mcl of cells. The optimal pulse duration was 4–5 ms. After the discharge, 1 ml of THB medium was added to the cuvette; the bacterial suspension was incubated for 1 h at 37°C and plated on THA with 10 µg/ml of erythromycin. The appearance of *E. faecium* L3-SARS transformants was monitored after 24 h.

Transcription of the *SarsS* Protein Gene Fragment Inserted in Bacterial DNA

The expression of mRNA was studied using real-time PCR (rRT-PCR) with reverse transcriptase using primers specific for the S-protein. Bacteria were grown in THB medium at 37°C for 18 h. *E. faecium* L3-SARS was cultivated with 5 µg/ml of erythromycin (Sigma, United States). Bacteria were washed three times in PBS by centrifugation at 3,500 rpm for 20 min and suspended in PBS. There was 10x concentrate used for mRNA analysis. Isolation of total RNA was carried out using the GeneJET RNA Purification Kit (Thermo Scientific, Waltham, United States). The isolated RNA was treated with 1 U/µl DNase (Invitrogen, Waltham, United States) after which one-step rRT-PCR was performed on a SFX96 thermocycler (BioRad, Hercules, United States) using HS-qPCR SYBR Blue master-mix (Biolabmix, Novosibirsk, Russia). *SarsS* specific primers K1 и K2 were used for analysis of SarsS protein gene expression and D-alanine-D-alanine ligase gene of *E. faecium* L3 as follows: Dal1и Dal2 as the normalizing gene.

Immunological Analysis

Immunization Schemes and Antibodies

To evaluate recombinant SarsS protein immunogenicity, mice were injected s. c. on the back two times at 3-week intervals with the 20-µg dose of protein per each immunization. The protein in 0.2 ml of PBS was emulsified together with 0.1 of Imject Alum (Thermo Scientific, United States) before the injection. Blood

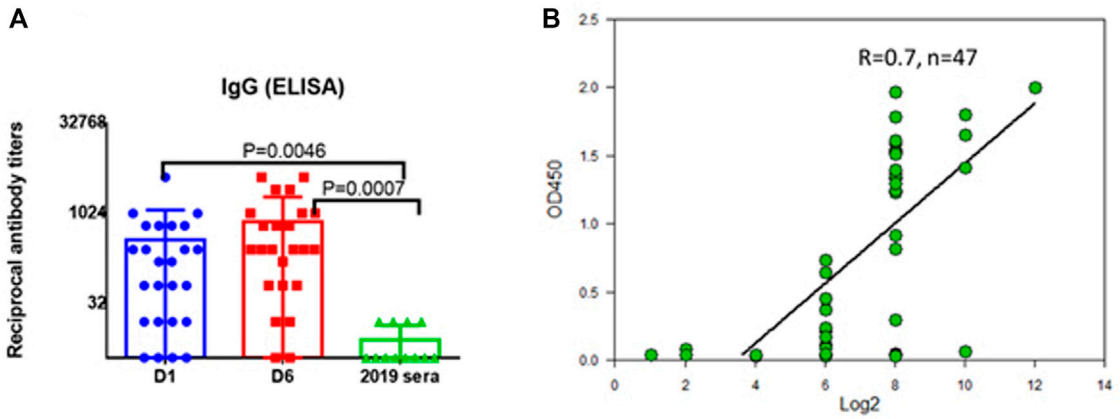

FIGURE 2 | Serum IgG to recombinant SarsS-protein in patients with confirmed SARS-CoV-2 infection. **(A)** D1—first day of hospital stay (n = 28), D6—sixth day of hospital stay (n = 28), 2019 sera were obtained from the patients examined in 2019 (n = 14). **(B)** 47 serum samples from patients with confirmed SARS-CoV-2 infection were studied using a commercial kit for detecting IgG antibodies to coronavirus "SARS-Cov-2 IgG Screen" (Imbian, Russia) in comparison with recombinant S1 protein. A high level of correlation was shown (Spearman's rank correlation coefficient = 0.7, n = 47) when detecting serum antibodies to SARS-CoV-2 using a recombinant protein and using a commercial kit. A high level of correlation was shown when serum IgG were detected using "SARS-Cov-2 IgG Screen" kit or recombinant S-protein, Spearmen r = 0.7. The positive value according to the screen data corresponded to 1:256 antibody titers obtained using S-protein.

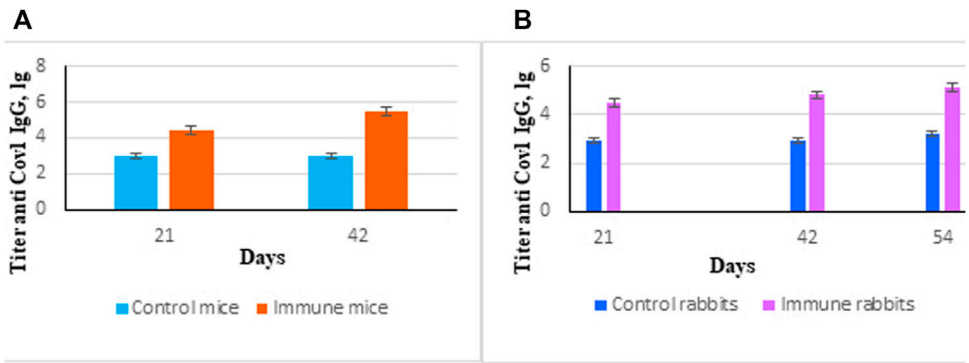

FIGURE 3 | Immune response induced by immunization of animals with recombinant protein SarsS. **(A)** Mice (n = 10) were immunized twice subcutaneously with the recombinant protein SarsS together with aluminum hydroxide as adjuvant. Control mice (n = 10) received aluminum hydrochloride as an adjuvant. Antibody titer was determined by ELISA as described in *Western Blotting*. **(B)** Rabbits (n = 3) were immunized three times subcutaneously with the recombinant protein SarsS together with aluminum hydroxide as vaccine adjuvant. Control rabbits (n = 2) received aluminum hydrochloride as an adjuvant. Antibody titer was determined by ELISA as described in *ELISA Assay*. Reciprocal antibody titers were characterized as log10 (ELISA) and presented as mean ± SEM. The *p*-values are obtained after comparison with control animals. Data were analyzed using the Student's t-test * - $p < 0.05$.

samples were taken from the submaxillary vein on the specified days or mice were bled 3 weeks after the last injection. The blood samples were centrifuged at 1,500×g for 10 min, and the collected sera were stored at −20°C.

To evaluate recombinant SarsS protein immunogenicity in rabbits, animals were injected i. c. into the sides three times at 3-week intervals. Three rabbits were immunized with the 120-μg dose of protein in PBS together with Imject Alum (Thermo Scientific, United States) in the ratio of 1:1 and in total volume of 1.0 ml and two control rabbits were immunized with PBS only with Imject Alum. Blood samples were taken from the ear vein of rabbits before the experiment and on the specified days. The collected sera were prepared and stored as described above.

The study used serum/plasma samples remaining from routine clinical tests of COVID-19 patients admitted to Vsevolozhsk Clinical Interdistrict Hospital, Leningrad Region, Russian Federation during March-April 2020. Positive SARS-Cov-2 results were identified by licensed laboratory tests (Imbian, Russia). The study employing the human sera was approved by the Local Ethics committee of the FSBSI "IEM" (protocol 3/20 from May 06, 2020). After receiving the approval of the Ethics Committee, the sera were handed over to the researchers none of whom had access to

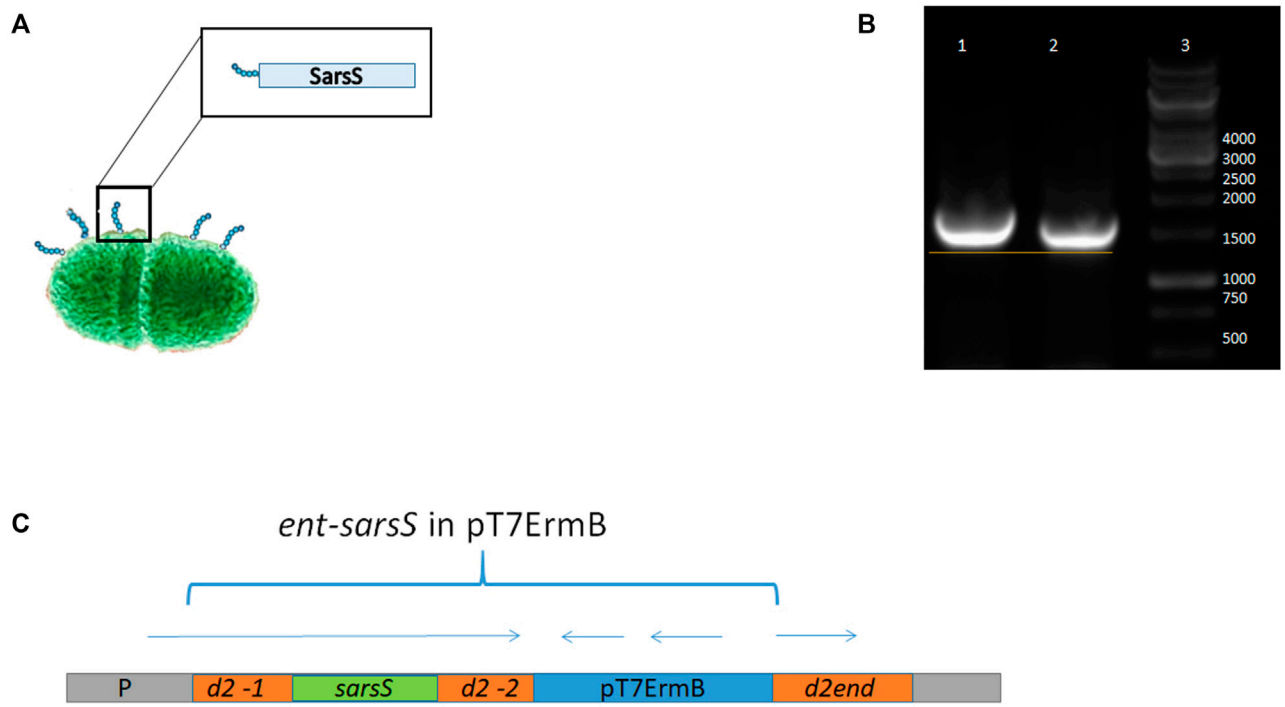

FIGURE 4 | Schematic representation of SARS-Cov-2 DNA fragment integration in enterococcal pili gene. **(A)** Putative enterococcal cell with pili protein containing S1 protein fragment. **(B)** PCR with DNA primers flanking the insert in the strain L3-SARS relatively to the appropriate region of the strain-recipient *E. faecium* L3. It can be seen that in the recombinant strain the DNA insert is slightly larger due to the insertion of SARS-Cov-2 DNA. **(C)** Scheme of the genomic region of enterococci after the integration of the plasmid *pentF-sarsS* into *E. faecium* L3 genome.

personal data of patients. As this is a retrospective study, informed consent was not required.

Monoclonal antibodies against spike glycoprotein S1 Cat. number: ATMA10164Mo were purchased from AtaGenix laboratories (Wuhan, China).

Western Blotting

For Western blotting bacterial lysates or SarsS solution were incubated with Laemmle buffer in the presence of β-mercaptoethanol at 95 °C for 5 min, followed by SDS-electrophoresis and transferred to nitrocellulose membrane for blotting (Sigma-Aldrich, United States). The membranes were blocked by 3% skim milk (Fluka, Germany)/PBS with 0.05% Tween 20 and incubated at 37°C for 60 min with primary monoclonal Anti-SARS-CoV-2 (S1) antibody (CR3022) (Atagenix laboratories, China) diluted 1:1,000 at the same buffer or serum obtained from COVID-19 patients diluted 1: 250 followed by 60 min incubation in 3% skim milk with horseradish peroxidase-conjugated anti-human secondary antibodies (1:2000, Sigma, United States). Immunoreactive bands were detected using TMB liquid substrate system for membranes (Sigma). Bacterial lysates or SarsS solution were incubated with Laemmle buffer in the presence of β-mercaptoethanol at 95°C for 5 min followed by SDS electrophoresis and transferred to nitrocellulose blotting membrane (Sigma-Aldrich). Membranes were blocked in 3% skim milk (Fluka, Germany)/PBS with 0.05% Tween 20 and incubated at 37°C for 60 min with Anti-SARS-CoV-2 (S1) (CR3022) primary monoclonal antibody (Atagenix laboratories, China) diluted 1:1,000 in the same buffer, or serum obtained from patients with COVID-19, diluted 1:250. Next, a 60-min incubation was carried out in 3% skim milk with secondary anti-human antibodies conjugated with horseradish peroxidase (1:2000, Sigma, United States). Immunoreactive bands were detected using the TMB Liquid Substrate Membrane System (Sigma, United States).

ELISA Assay

ELISA assay was done as described earlier (Gupalova. et al., 2019). Briefly, Maxisorb 96-well plates (Nunc; Denmark) were coated overnight at 4°C with 0.25 μg/ml of protein S in 0.1 M sodium carbonate buffer pH 9.3. A series of twofold dilutions of the sample (100 μl) was added to duplicate wells and incubated for 1 h at 37°C. Between the different stages, the plates were washed with blocking buffer (0.05% Tween-20 in PBS). The same buffer was used for serum and reagents dilution. HRP-labeled goat anti mouse IgA or IgG antibodies (Sigma) were added (100 μl/well). After incubation at 37°C for 1 h, the plates were developed with 100 μl/well TMB substrate (BD Bioscience). A color was detected after 20 min of incubation after stopping the reaction with 30 μl of 50% sulfuric acid. The endpoint ELISA titers were expressed as the highest dilution that yielded an optical density at 450 nm (OD450) greater than the mean OD450 plus 3 standard deviations of negative control wells.

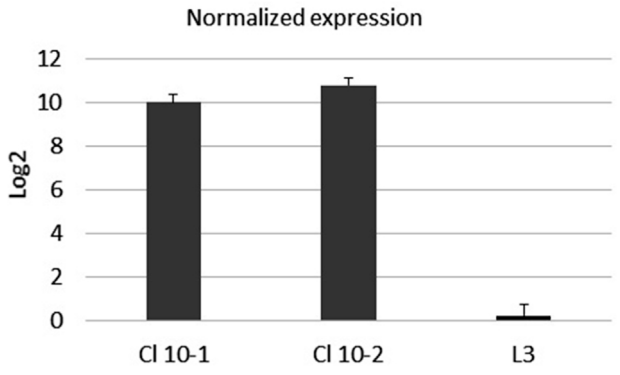

FIGURE 5 | The mRNA expression level of SarsS-protein gene fragment in the culture of *E. faecium* L3. Data were analyzed using the comparative threshold cycles (ΔΔCt) method, normalized to D-alanine-D-alanine ligase gene of L3, and presented as the fold changes in gene expression of S-protein modified L3 relative to the pure L3. Black bars represent the averaged data obtained based on two L3-SARS cultures, the bar at the right represents the averaged data obtained with pure *E. faecium* L3. The average Ct values were 13.5 when RNA from modified L3-SARS or *E. faecium* L3 was amplified using primers to the D-alanine-D-alanine ligase as a housekeeping gene. When using primers specific to S-protein, RNA isolated from modified *E. faecium* L3 cultures gave average Ct values of 28.5, and for pure *E. faecium* L3 average Ct values were more than 40 which indicated the absence of amplification.

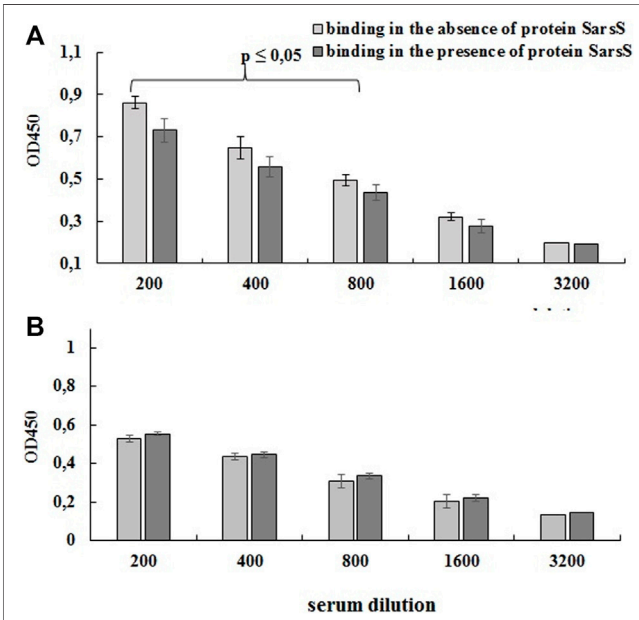

FIGURE 6 | Detection of protein SarsS on the surface *of E. faecium* L3 by ELISA. A serum of patients recovered from SARS-CoV-2 was analyzed by competitive ELISA in the 96-well plates covered with L3-SARS **(A)** and *E. faecium* L3 **(B)**. The results allowed evaluating the effect of SARS-Cov-2 specific serum to bind modified *Enterococcus* strain after the preliminary incubation with recombinant SarsS protein. The figure shows the data of one of the three independent experiments, the conclusions from which completely coincided. Each point is the average of four measurements. Data presented as mean ± SEM. Data were analyzed using the Student's t-test.

Detection of the Surface Display of the Recombinant SarsS Protein on *E. faecium* Cells

To determine the localization of protein SarsS on the surface of bacteria, we compared the results of sandwich and competitive ELISA on biofilms of the original and modified *Enterococcus*. For this purpose, we used the human polyclonal sera containing IgG specific to the S1 protein of the SARS-CoV-2.

The procedure for preparing enterococcal biofilms was carried out according to (Tabenski et al., 2014). Biofilms of original *E. faecium* L3 or genetically modified *E. faecium*.

L3-SARS were grown in 96-well plates in sterile THB with 0.5% yeast extract by incubation at 37°C for 24 h. To reduce the nonspecific binding of human serum IgG to the *E. faecium* L3, the serum was pre-incubated with bacteria at a concentration of 10^9 CFU/ml and incubated at 37°C for 60 min with shaking. The bacteria were separated by centrifugation at 2000 *g* for 10 min and the supernatant was analyzed in ELISA with and without SarsS protein treatment. The supernatant was divided into two samples, one of which was supplemented with protein SarsS at a concentration of 4 μg/ml, and the other with an equal volume of PBS. Both samples were incubated at 37°C for 60 min with shaking. Then each sample was analyzed in ELISA as described in paragraph *Western blotting* in 96-well plates with the biofilms of the original (*E. faecium* L3) and modified (L3-SARS) strains on the bottom.

Animal Procedures

Female inbreed Balb/c mice were obtained from the laboratory animal nursery "Rappolovo" (Leningrad Region, Russia) and were used in experiments at the age of 10 weeks. Female inbreed rabbits (2.5 kg weight) were obtained from the laboratory animal nursery "Rappolovo" (Leningrad Region, Russia). Mice and rabbits were housed under standard laboratory conditions with food and water ad libitum. The experiments were developed in accordance with EU Directive 2010/63/EU for animal experiments, approved and carried out according to guidelines and under the supervision of the local biomedical ethics committee (minutes of the meeting 1/21 dated January 28, 2021).

Statistical Analyses

The results are presented as the mean ± standard deviation of the mean (SEM). The statistical data analysis was performed using the Student's t-test and *p*-values <0.05 were considered as statistically significant. Statistical data processing was performed using the Statistica 12.0 software package (StatSoft, Inc. Tulsa, Oklahoma). For statistical analysis, antibody titers were expressed as the log10 of the final reciprocal dilution.

Bioinformatics Analyses

DNA and putative protein analysis were performed employing BLAST NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ExPASy (http://www.expasy.org program) packages available in public domains. DNA primer design was accomplished by Primer 3.0 computer program. Protein sequence analysis for the presence of the B-cell and T-cell epitopes was preformed

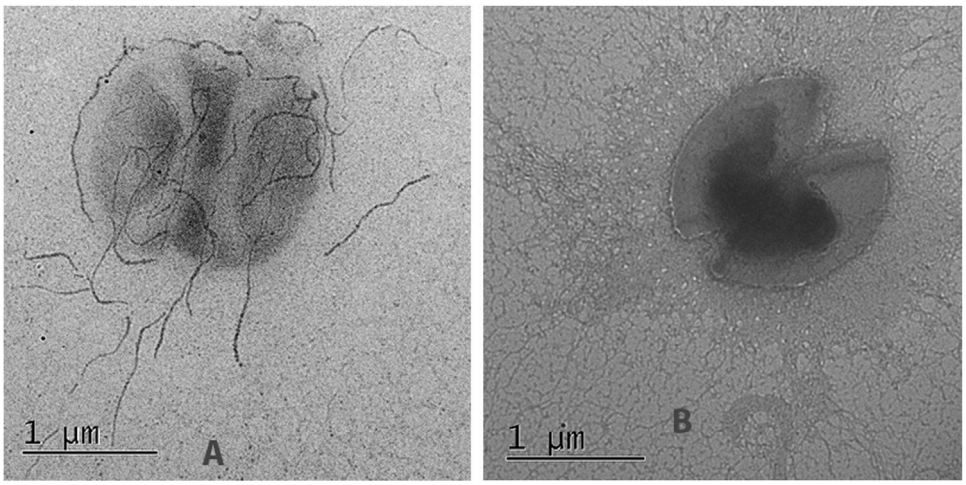

FIGURE 7 | Immunoelectron microscopy of the original (*E, faecium* L3) and genetically modified strain (L3-SARS) after detection with polyclonal serum from patients with COVID-19 with the following immunogold labeled goat IgG conjugated to 18 nm gold particles as described in Section 2.8. **(A)** strain L3-SARS. **(B)** *E. faecium* L3.

employing IDEB (Immune Epitope Database) analysis and data base resource tool (https://www.iedb.org/).

Electron Microscopy

Immunoelectron microscopy was aimed to study the structure of *E. faecium* L3 pili with expression of viral proteins. Bacteria were grown in LB (lysogeny broth, VWR Life Science Products Amresco, Solon, United States) medium at 37°C for 18 h. *E. faecium* L3-SARS was cultivated with 5 μg/ml of erythromycin. Bacteria were washed three times in PBS by centrifugation at 3,500 rpm for 20 min and suspended in 0.1 M NaCl. There was 10x concentrate used for immune-electron microscopy. The source of primary antibodies was human polyclonal serum containing IgG specific for S1 of Sars-Cov-2 which were previously adsorbed twice on pure L3 culture in a ratio of 1:10 in order to avoid nonspecific binding. Immunogold labeling was performed using goat IgG conjugated to 18 nm gold particles (1 mg/ml; Jackson ImmunoResearch Laboratories, West Grove, United States). The bacterial culture samples were applied to the grids for transmission electron microscopy (FF400-AU Formvar Support Film 5–6 nm thick on Square, 400 mesh Gold Grid, Electron Microscopy Science, Hatfield, PA) by the method for liquid suspensions ("drop on the grid") followed by incubation for 2 min at room temperature. Then the grids were fixed for 1 min in 2.5% paraformaldehyde in PBS. Blocking was performed with 0.1% gelatin in PBS for 1 h. Primary antibodies were diluted in 2% BSA/PBS, incubation lasted for 1 h. Secondary antibodies were diluted 1:20 in 2% BSA/PBS, and the samples were incubated for 1 h. For contrasting, 1% uranyl acetate was used in a drop of 20 μL (20 s). To compact the resulting films, they were covered with a layer of nanocarbon. Electron microscopy was performed on a JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan). Photos were taken with digital cameras: bottom port - Gatan Ultrascan 4,000 16 Mpix, 4 × 4 K, 16 bit; side port - Gatan Erlangshen 500 1.4 Mpix, 1.3 × 1 K, 12 bit, 15 fps (Gatan, Pleasanton, United States).

RESULTS

Bioinformatics Analysis

The technology of making live enterococcal vaccine a candidate requires insertion of the heterologous gene fragment in the range of about 500 bp in order to generate a chimeric protein capable of assembly into pili on the surface of bacteria. In order to select the region of the spike protein gene of that size encoding for immunogenic S-protein domains, we have used several protein analysis tools including ExPASy and IDEB on-line platforms. Bioinformatics analysis of S protein sequence obtained from NCBI database revealed an extended B-cell linear epitope of 52 amino acids NFNGLTGTGVLTESNK KFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSF in the area of close proximity of RBD domain with several shorter epitopes TVCGPKKSTNLVKNKCV, and VITPGTNTSN are in the same region (**Figure 1**). This very area of S protein contained several putative T-cell epitopes (data not shown). The DNA sequence corresponding to this S protein area was synthesized and used to make the expression plasmid *pQE-sarsS* and suicidal plasmid *pentF-sarsS* which was constructed for insertion into the bacterial genome (**Supplementary Figure S1**). The sequence was deposited into GeneBank (Spike_SARS_incertion OL447006-1 https://www.ncbi.nlm.nih.gov/nuccore/OL447006.1/).

Development and Analysis of Recombinant Protein SarsS

Construction of the recombinant plasmid pQE-sarsS was performed as described in *Development and cloning of a fusion gene entF- sarsS*. Recombinant DNA was obtained after cloning of the selected fragment of SARS-Cov-2 DNA into expression plasmid pQE-30. Resultant plasmid designated as *sarsS* was used for transformation of the producer strain of *E. coli* M15 with production of recombinant protein SarsS.

The molecular weight of recombinant protein SarsS was determined as (24, 5 ± 0.5) kDa. (**Supplementary Figure S2A**). This size corresponded with the predicted size of the amino acid sequence of 168 amino acid residues belonging to SARS-CoV-2 linked to 21 amino acid residues encoded by the vector plasmid pQE-30. The analysis of the amino acid sequence of recombinant protein SarsS was also confirmed by MALDI TOF/TOF.

Purified recombinant protein SarsS was also able to bind IgG obtained from the patients with Covid-19 (**Supplementary Figure S2B**). The recombinant protein SarsS was subjected to polyacrylamide gel electrophoresis (PAAG) and stained with (**Supplementary Figure S2A**) Amido Black; the same protein was transferred to nitrocellulose membrane (**Supplementary Figure S2B**) and blotted with IgG obtained from COVID-19 patients as described in *Immunization Schemes and Antibodies* and *Western Blotting*. The molecular weight of the protein bands stained in immunoblotting coincided with the weight of protein SarsS1 and its dimer and was approximately equal to 25 and 50 kDa.

The correspondence of the recombinant protein to its natural version was investigated by immunoblotting with sera of people who had undergone coronavirus infection. The recombinant protein SarsS was found to interact with antibodies in human immune serum obtained from the COVID-19 patients employing ELISA (**Figure 2A**). Results of detection of the antibodies against SARS-Cov-2 with SarsS protein nicely correlated with the results of the assay with commercial coronavirus detection system "SARS-Cov-2 IgG Screen" (Imbian, Russia) (**Figure 2B**).

Purified protein SarsS was used for immunization of mice and rabbits as described in the *Materials and Methods* section. Specific IgG started to appear on Day 21 with a small additional increase at Day 42 (**Figures 3A,B**). This protein was used in ELISA as an antigen to determine the level of antibodies in serum and nasal lavages as well as for subcutaneous immunization to obtain specific hyperimmune serum. Twofold subcutaneous immunization of mice or threefold immunization of rabbits with protein SarsS in the presence of an adjuvant showed a significant amount of the S-protein specific IgG in blood serum of both types of animals. This data demonstrated that SarsS protein was able to generate specific SARS-Cov-2 immune response after subcutaneous immunization which was reflected in IgG production and immunogenicity of the recombinant protein SarsS.

However, there was only a little increase of S specific IgA in blood serum of mice which might reflect the way of antigen delivery (**Supplementary Figure S3**).

Development and Cloning the Fusion Gene *entF-sarsS*

Recombinant plasmid DNA *pentF-sarsS* was constructed as described in *Development and cloning of a fusion gene entF- sarsS*.

The plasmid was sequenced employing DNA primers presented in **Table 1**. Results of DNA sequencing demonstrated that *SarsS* was inserted in frame with the sequence of *E. faecium* MPP gene. Thus, p*entF-sarsS* plasmid DNA contained inserts of the *sarsS* gene fragment and the flanking fragments of the MPP gene necessary for recombination into the *E. faecium* L3 genome.

The scheme of enterococcal clone design is presented in **Figure 4**.

Electro Transformation of Enterococci

Fourteen transformants were obtained after electroporation of enterococci and delivering of the integrative plasmid *pentF-sarsS*. All of them were tested in a PCR reaction with primers K1 and K2. To prove the integration of the *pentF-sarsS* plasmid DNA into the *Enterococcus* genome, the DNA isolated from all clones was amplified with primers B1 and K2. B1 is a primer for the *Enterococcus* MPP gene; K2 is a primer for the insert. Only two clones in the PCR reaction showed the presence of a necessary fragment, the size of which was equal to the sequence between primers B1 and K2 (**Table 1**). DNA sequencing confirmed that integration of the plasmid *pentF-sarsS* took place exactly as it was originally designed–in the homology region of MPP d2-1 which is located upstream *SarsS* (**Supplementary Figure S4**). One of the *Enterococcus faecium* L3 clones having the SarS protein gene fragment of SARS-CoV-2 in frame with enterococcal MPP protein gene was selected as a vaccine strain for further research and designated as L3-SARS.

Study of the Expression of DNA Fragment of the Spike Protein S in the Composition of L3-SARS

We studied the expression of the inserted viral gene fragment *sarsS* at the stage of mRNA synthesis, SarsS protein production, and its localization in a bacterial cell.

mRNA Expression

The expression of mRNA in real-time PCR with reverse transcriptase (rRT-PCR) was examined with primers specific for the S-protein, according to the procedure described in *Transcription of the SarsS protein gene fragment inserted in bacterial DNA*. To confirm the expression of the inserted *sarsS* gene fragment in bacterial DNA, we studied the expression of mRNA using real-time reverse transcriptase PCR (rRT-PCR) with *SarsS* specific primers K1 и K2. (**Table 1**). Results of rRT-PCR demonstrated a dramatic increase of amplification of the *SarsS* sequence relative to the control (**Figure 5**).

These results assured that *sarsS* DNA in *E. faecium* L3 genome is transcribed together with the target gene in *Enterococcal faecium* genome.

SarsS Protein Expression in L3-SARS

In order to prove that the spike protein gene fragment fused to the enterococcal MPP gene is properly translated in the strain L3-SARS we lysed the recombinant bacteria and tested the cell lysate in an immune blotting procedure. For this purpose both modified and original *E. faecium* L3 strains were grown overnight, washed, disrupted, and tested in 10% PAAG and Western blotting with S1-specific monoclonal antibodies (**Supplementary Figure S5**).

Only L3-SARS cells lysate generated a band around 80 KDa which was binding the monoclonal antibodies. This band corresponded to the expected size of the MPP protein (**Supplementary Figure S5**) which was slightly larger than the original MPP because the coding area of the chimeric gene exceeded the size of the original gene of the major pili protein. As expected, monoclonal antibodies against S protein did not bind to *E. faecium* L3.

Detection of S Protein Antigenic Epitopes on the Surface of Enterococci

For this purpose, bacterial cells on the bottom of the immunological 96 wells plates were probed with the human serum which was preliminarily adsorbed with purified SarS recombinant protein as described in Section 2.3.4. As shown on **Figure 6A** preliminary incubation of the serum with recombinant protein SarsS significantly decreased the level of the binding of antibodies with L3-SARS. On the contrary, when *E. faecium* L3 was tested in the same experiment we could not determine any difference (**Figure 6B**). This data show that SARS-CoV-2 specific antigens are expressed on the surface of recombinant bacteria L3-SARS.

Electron microscopy of the strain L3-SARS depicted numerous chains of more than 10 molecules interacting with the gold label. Interestingly some of the label accumulates just on the cell surface depicting differential expression of the pili protein changed by genetic manipulations (**Figure 7A**). As expected, *E, faecium* L3 was free from the specific interaction with IgG from COVID-19 patients (**Figure 7B**). Immune electron microscopy data shows that a fragment of spike protein from SARS-Cov-2 not only expresses on the surface of enterococcal recombinant strain but is also capable of being part of a properly assembled enterococcal pili. This finding makes the strain L3-SARS an interesting vaccine candidate due to the easy access of pili to the host immune system.

DISCUSSION

Delivery of the antigens to the mucous membranes as part of the viral or bacterial vector is a reasonable approach, since it overcomes several problems inherent in parenteral vaccines. Intranasal or oral delivery of the antigen is convenient, non-traumatic, and economical. The oral antigen delivery system usually is able to stimulate an intense mucosal immune response. This distinguishes the mucosal route from parenteral vaccination (Taghinezhad-S et al., 2021).

Various pre-clinical and clinical studies provided the evidence that oral immunization offers several advantages over other ways of immunizations. This includes better stimulation of gut-associated lymphoid tissue (GALT), enhanced production of anti-viral IgA, effective induction of mucosal immune responses, decreased risk of contamination, cost-effectiveness, and easy self-administration or administration to animals (Qiao, et al., 2009; Wang et al., 2012; Mohseni et al., 2020). Antigen production by live probiotic bacteria capable of multiplying in the gut and affecting larger mucosal area for a prolonged period of time is an additional advantage of probiotic vaccines.

All these data provide a substantial amount of information advocating the usage of probiotic vaccines as defense against viral pathogens. What is important, oral vaccination, compared to the nasal route, can significantly increase DC activation, specific IgA production, $CD8^+$ T-cell induction, and cross-protection against viral challenge *in vivo* (Yang et al., 2016). Additionally, *in vivo* studies proved that oral intake of recombinant LAB can provide higher neutralizing antibody activities compared to intraperitoneal injection (Sim et al., 2008; Tang and Li, 2009).

It is also important to notice that the state and condition of microbiota provide an additional defense against the viral pathogens on mucosal surfaces which make probiotic bacteria extremely useful as factors positively influencing the microenvironment. Clinical studies and human trials suggest that several probiotic strains including L. rhamnosus GG, L. casei, L. plantarum, L. casei strain Shirota, B. lactis Bb-12, and B. longum were able to reduce the prevalence of upper respiratory infections, common cold, flu-like symptoms, and antibiotic-associated diarrhea by 40–70% (Rautava et al., 2009; Smith et al.,. 2013; King et al., 2019). Furthermore, probiotic strains such as L. reuteri ATCC 55730, L. paracasei, L. casei 431, L. fermentum PCC, and B. infantis 35,624 were pivotal in producing immunomodulatory responses during various infections (Oliva et al., 2012; Pu et al., 2017; Zhang et al., 2018; Villena et al., 2021).

The hostile environment of the gastrointestinal tract, which includes the stomach extreme pH and the intestinal protease-rich environments, can severely affect the immunogenicity of ingested antigens. These characteristics have made the generation of efficient oral vaccines extremely challenging, due to the difficulty of finding appropriate antigen delivery systems and adjuvants that efficiently stimulate mucosal immunity.

Integration of vaccine antigens into the genome of probiotic microorganisms provides additional advantages to probiotic vaccines. The beneficial effects of the administration of probiotics complement the effect of specific immune stimulation and enhance the mucosal immunity (Singh and Rao 2021). The aim of the present study was to construct a novel vaccine candidate in a form of live bacterial vaccine with a gene fragment encoding to evaluate the S-specific immune response after the oral administration of a live vaccine. For this purpose, it was necessary to select a bacterial vector, viral fragment with immunogenic features, and to generate bacteria, capable of expressing SARS-Cov-2 epitopes on the surface.

Previously we developed and successfully used a method for inserting gene fragments of pathogenic streptococci into the *E. faecium* L3 genome to obtain live antibacterial vaccines (Gupalova et al., 2018; Gupalova et al., 2019). *E. faecium* L3 is a well-studied probiotic bacteria with good clinical evidence of safety and usage in the case of chronic gastro-intestinal diseases, *H. pylori* infections, and multiple sclerosis (Lo Skiavo et al., 2013; Baryshnikova et al., 2015; Abdurasulova et al., 2016; Suvorov, 2020).

The method designed to incorporate streptococcal DNA fragments into the enterococcal gene encoding the major pili protein and thus express the foreign protein on the surface of the probiotic bacterium rely on the permanent integration of the suicidal plasmid into bacterial genome. It is well established that

Gram-positive bacteria, including *Streptococcus pneumoniae* and *E. faecium,* assemble long filamentous pili on their surface through which they adhere to host cells (Nallapareddy et al., 2006; Spraggon et al., 2010; Khare and Narayana, 2017).

For example, pneumococcal pili are formed by a backbone, consisting of the repetition of the major component RrgB (or MPP in *Enterococcus*)—main repetitive structural domain of the native pilus with LPXTG motive in C terminus of the protein. This motive is necessary for the attachment of the protein subunit with N terminus of the next molecule in the chain of the pili. In the present study, part of the MPP gene with the d2 domain was replaced with a part of the S glycoprotein (spike) if modification of the central part of the gene will not interfere with the protein chain assembly. The SARS-Cov-2 S glycoprotein (~141 kDa and ~1,270 amino acids) is responsible for the viral attachment to the human cell receptor ACE-2. We have selected an immunogenic part of the protein, which is located in an area of close proximity with ACE-2 binding domain and inserted 501 bp DNA fragment encoding for this region into the probiotic genome. Analysis of the resultant recombinant enterococcal strain by PCR and following DNA sequencing revealed the presence of the spike DNA sequence in enterococcal genome (**Supplementary Figure S4**). The strain, selected on the agar plates with antibiotic, was highly stable and did not lose the insert after 48 generations in media without antibiotic. In the organism of mice after feeding it stayed more than a week which proved that the strain L3-SARS was able to multiply in the animal gut (data not shown).

The spike gene fragment cloned in the expression plasmid was successfully tested for the ability to induce specific immunity in two different types of animals–mice and rabbits. In addition, the protein SarsS was specifically interacting with human IgG of the COVID-19 patients. This DNA sequence cloned in probiotic genome in frame with the pili protein gene directed synthesis of S-specific mRNA and the appearance of the surface protein capable of binding to the monoclonal antibodies against S-protein epitopes. Surface display testing of the resultant enterococcal strain L3-SARS revealed that the recombinant bacteria possessed the ability to specifically bind IgG from COVID-19 patients. Interestingly the cells of the original enterococcal strain *E. faecium* L3 were also binding human immunoglobulins. It is known that the natural immune response of mammals to microbiota includes T-independent induction of polyspecific antibodies, which are present in blood serum and act as a regulator of commensal microbiota including enterococci (Pabst et al., 2016; Zeng et al., 2016).

After the co-incubation of the antibodies with the SarsS protein, their binding to the *E. faecium* L3 and L3-SARS revealed significant differences. Indeed, pre-incubation with SarsS protein significantly reduced the binding of immune serum IgG to the L3-SARS surface relatively to control (**Figure 6**).

SARS-Cov-2 specific antigens on the surface of L3-SARS were determined by electron microscopy (**Figure 7A**) which demonstrated the proper assembly of the chimeric pili molecules on the surface of bacteria.

Taken together the results of the study allow concluding that selected fragments of SARS-Cov-2 DNA were able to direct synthesis of immunogenic protein that was expressed by the strain of *E. faecium*. Further experiments of the strain L3-SARS regarding its ability to generate the specific humoral and T-cell response are required for evaluating the possibility using this strain as a candidate for oral vaccine against SARS-Cov-2.

CONCLUSION

The aim of the study was to generate a novel probiotic vaccine candidate for mucosal immunization against SARS-Cov-2. The fragment of SARS-Cov-2 DNA encoding for S1 protein was cloned in the *E. coli* expression vector, isolated as recombinant protein, and tested for immunogenicity in mice and rabbits. This DNA fragment was also inserted in *E. faecium* probiotic genome. The resultant strain L3-SARS was expressing SARS-Cov-2 specific epitopes on the surface able to react with specific IgG from the COVID-19 patients and S1 specific monoclonal antibodies. Further experiments of the strain regarding its ability to generate the specific humoral immune response, T-cell response, and anti-SARS-Cov-2 protection will follow.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/nuccore/OL447006.1/.

ETHICS STATEMENT

The study employing the human sera was approved by the Local Ethics committee of the FSBSI "IEM" (protocol 3/20 from May 06, 2020). After receiving the approval of the Ethics committee, the sera were handed over to the researchers, none of which did not have access to personal data of patients. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The experiments were developed in accordance with EU Directive 2010/63/EU for animal experiments, approved and carried out according to guidelines and under the supervision of the local Biomedical ethics committee (Minutes of the meeting 1/21 dated January 28, 2021).

AUTHOR CONTRIBUTIONS

Conceptualization, AS and GL; methodology, TG, GL, TG, and TK; formal analysis, OK and YD; investigation, TK, EB, GL, OK, YD, and IK; writing—original draft preparation, TG, GL, and YD; writing—review and editing, AS and GL; visualization, GL, YD, and OK; supervision, AS. All

authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.807256/full#supplementary-material

Supplementary Figure S1 | DNA sequence of the *sarsS* gene used for cloning.

Supplementary Figure S2 | PAGE analysis of recombinant protein SarsS and immunoblotting with antibodies from COVID-19 patients. The recombinant protein SarsS was subjected to polyacrylamide gel electrophoresis (PAGE) and stained with (lane A) Amido Black; the same protein was transferred to nitrocellulose membrane (lane B) and blotted with IgG obtained from COVID-19 patients as described in *Immunization schemes and antibodies* and *Western blotting*. 1) SarsS protein. 2) Protein molecular weight marker. 3 –Immunoblotting of SarsS being transferred on the nitrocellulose with IgG obtained from COVID-19 patients.

Supplementary Figure S3 | Immune response to subcutaneous immunization of mice with protein SarsS on Day 42 after the first injection.

Supplementary Figure S4 | Chromosomal DNA region of *Enterococcus* with the nucleotide sequence of the integrated plasmid DNA *pentF-sarsS* .DNA primers B1 and K2 used for sequencing are highlighted in bold.

Supplementary Figure S5 | Detection of protein SarsS in lysate of *E. faecium* L3 by Western blotting. Enterococcal proteins were separated by electrophoresis in 10% PAGE, transferred to a nitrocellulose membrane and blotted with commercial monoclonal antibody (AtaGenix Laboratories, Wuhan, China), specific to S1 protein of SARS-CoV-2.

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REVIEW
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Role of Butyrate, a Gut Microbiota Derived Metabolite, in Cardiovascular Diseases: A comprehensive narrative review

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Cardiovascular diseases (CVD) are major causes of death worldwide. Recently, new roles for intestinal microbiota in pathology and treatment of CVD have been proposed. Butyrate, a bacterial metabolite, is synthesized in the gut and performs most of its functions in there. However, researchers have discovered that butyrate could enter to portal vein and interact with various organs. Butyrate exhibits a broad range of pharmacological activities, including microbiome modulator, anti-inflammatory, anti-obesity, metabolic pathways regulator, anti-angiogenesis, and antioxidant. In this article we review evidence supporting a potentially therapeutic role for butyrate in CVD and the mechanisms and pathways involved in the cardio-protective effects of butyrate from the gut and circulation to the nervous system. In summary, although butyrate exhibits a wide variety of biological activities in different pathways including energy homeostasis, glucose and lipid metabolism, inflammation, oxidative stress, neural signaling, and epigenetic modulation in experimental settings, it remains unclear whether these findings are clinically relevant and whether the molecular pathways are activated by butyrate in humans.

Keywords: gut microbiota, butyrate, cardiovascular diseases, epigenetic modulation, antioxidant

INTRODUCTION

Cardiovascular diseases (CVD) are disorders that affect the heart and blood vessels mainly including heart failure (HF), stroke, atherosclerosis, and hypertension (Ahmad et al., 2019). CVD are at the top of the life-threatening ailments list globally (Hulten et al., 2017). Based on the report of the World Health Organization (WHO), 17.9 million deaths were attributed to CVD in 2019, which accounted for 32% of all deaths (Roth et al., 2020).

Despite advances in primary prevention, CVD prevalence has risen in recent years (Tsivgoulis et al., 2018). The proven conditions that increase the risk of CVD including hypertension, dyslipidemia, obesity, and insulin resistance (IR) are concomitantly increased with CVD (Roth et al., 2020). In order to improve CVD prevention and treatment, it is essential to investigate unknown parts of the pathophysiology as well as to identify novel agents affecting risk factors (Singh et al., 2016; Warmbrunn et al., 2020). Dysbiosis of gut microbiota is one of the newest factors which is

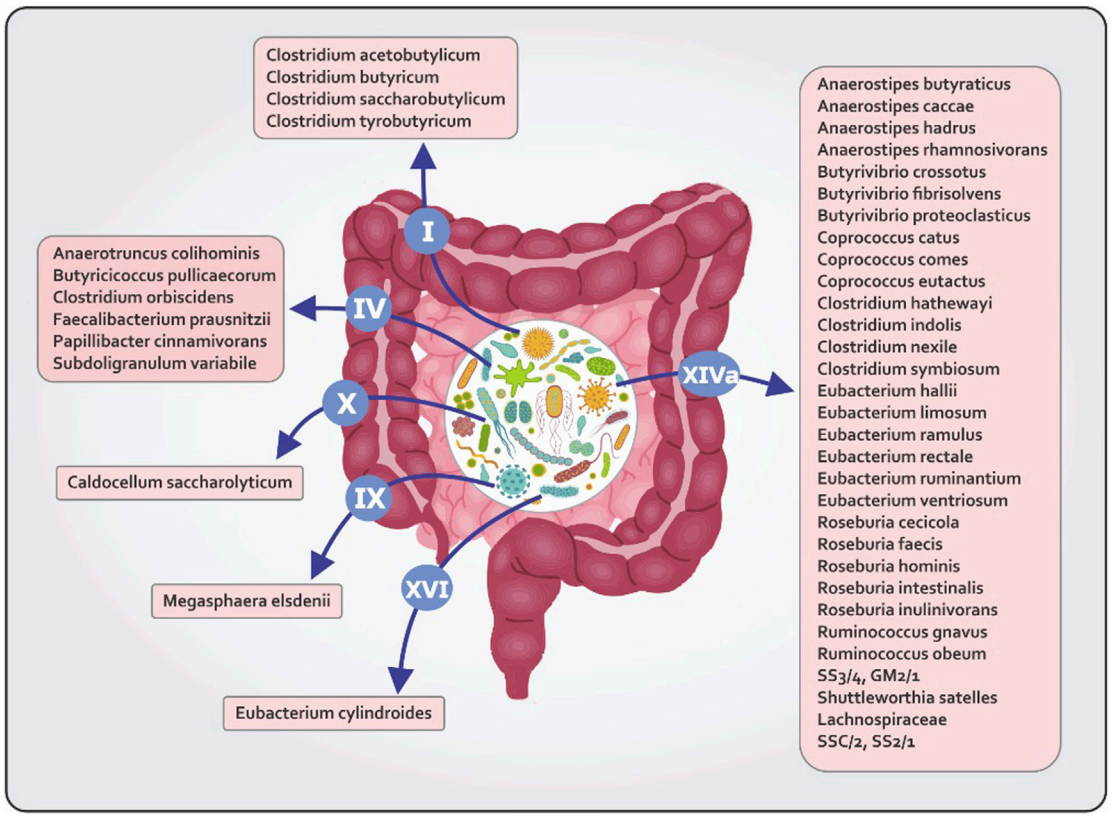

FIGURE 1 | All butyrate producing bacteria in different clostridial clusters.

known to be involved in the development of CVD (Lau et al., 2017). Dysbiosis is characterized as alterations in microbial composition and their metabolites (Serino et al., 2014). An increasing body of evidence in CVD indicates alterations in microbial composition and their metabolites that may play a role in the pathogenesis and progression of these diseases (Brown and Hazen, 2015; Witkowski et al., 2020). The gut microbiota produces a wide variety of metabolites as a result of the anaerobic fermentation of undigested foods (Warmbrunn et al., 2020). Short-chain fatty acids (SCFAs) including acetate, propionate and butyrate, are main metabolites that may provide important protection.

Butyrate is a four-carbon SCFA, mainly known as a fuel for colonocytes. In addition to dietary fibers especially resistant starch, which is an indirect source of butyrate, some types of cheese, butter, and milk also contain small amounts of butyrate. Researches showed that butyrate can absorb into the portal vein and interact with the host body's important processes like glucose homeostasis, lipid metabolism and gut inflammation. Several *in vitro* and *in vivo* studies have demonstrated that butyrate exerts anti-inflammatory, anti-oxidant, anti-obesity and metabolic regulation effects. The aim of the present study was to comprehensively review the therapeutic efficacy of butyrate in CVD as well as the mechanisms of action of butyrate on CVD risk factors.

BUTYRATE PRODUCING BACTERIA AND ALTERATIONS IN CVD

Gut microbiota is made up trillions of microorganisms including bacteria, viruses, fungi and protozoa (Ley et al., 2006). A variety of dietary substrates are used by these microbes to produce a range of metabolites, some of which are beneficial to the host (Scott et al., 2013). The production of butyrate is widespread among diverse phyla of human colon (Louis and Flint, 2009). Firmicutes and *Bacteroides* are the main butyrate producing phyla (Chen et al., 2020). The identification of butyrate producing spices has been done using sequence based detection algorithms such as metagenomics and 16S ribosomal RNA sequencing (Louis and Flint, 2009). All butyrate-producing bacteria are shown in **Figure 1**. The most prominent butyrogenic bacteria groups are *Faecalibacterium prausnitzii*, *Butyrivibriocrossotus* and *Roseburia intestinalis* which several studies have reported the depletion of these bacteria in atherosclerosis (Chen et al., 2020). Kasahara et al. demonstrated that the abundance of Roseburia intestinalis is negatively correlated with the size of atherosclerotic lesions in the mouse model of atherosclerosis (Kasahara et al., 2018). In that study, when Roseburia intestinalis was taken along with a high-fiber diet, aortic atherosclerotic plaques were reduced in size. This study suggests that the butyrate, a microbial metabolite, mediates these effects (Kasahara et al., 2018). A research conducted by zeng

et al. illustrated that bacteria with capacity of butyrate production, Lachnospiraceae and Ruminococcaceae, were depleted in individuals at a high risk of stroke. Fecal butyrate concentrations also were low in these people (Zeng et al., 2019). Additionally, metagenomes analysis of individuals with symptomatic carotid atherosclerosis showed that gene expression of butyrate-synthesizing enzyme (butyrate-ace- toacetate CoA-transferase) were inversely correlated with C reactive protein (CRP) levels (Karlsson et al., 2012).

Several studies have reported that patients with HF have a decrease in butyrate-producing bacteria, especially, Lachnospiracea and Ruminococcacea families (Trøseid, 2020). Remarkably, reduction of the butyrate-producing Eubacterium Halli and Lachnospiracea is correlated with increased inflammation, severity of disease, heart damage and mortality (Kummen et al., 2018). Moreover, there is evidence that dysbiosis is related to low butyrate production in different HF cohorts (Trøseid, 2020). Likewise, animal and human studies reported decrease in bacteria with capacity of butyrate production in hypertension (Mell et al., 2015; Yang et al., 2015; Gomez-Arango et al., 2016; Yan et al., 2017). According to these studies, amount of butyrate producer bacteria is negatively associated with blood pressure (BP) (Santisteban et al., 2017).

Overall, the evidence suggests that decrease in butyrate-producing bacteria abundance, down regulation of genes involved in butyrate synthesis, and low butyrate levels are associated with sensitivity of developing CVD.

MECHANISMS OF ACTION OF BUTYRATE IN CVD AND CVD RISK FACTORS

Major Mechanisms

The key aspects of butyrate mechanism of action can be listed as follows. First, butyrate has reported to be an epigenetic modifier by acting as a histone deacetylases (HDACs) inhibitor (Chang et al., 2014). HDACs are chromatin-modifying enzymes that alter genes transcription accessibility by removing acetylate from histones and non-histone proteins (Shakespear et al., 2011). The abnormal regulation of gene expression underlies many human metabolic disorders such as CVD. By inhibiting HDACs, butyrate causes hyper-acetylation of transcription factors and regulates gene expression patterns (Cleophas et al., 2019).Second, butyrate influences cellular responses by binding and activating specific receptors named free fatty acid receptor 2 (FFAR2) and FFAR3 (Previously these receptors were called G protein-coupled receptors (GPRs), GRP 43 and 41 respectively) (Brown et al., 2003).

Butyrate can contribute in widespread cardiovascular-related functions through inducing intracellular signaling pathways by interacting with FFARs on target cells or causing epigenetic changes by inhibiting HDAC.

Butyrate as a PPARs Agonist

Butyrate is known to be a pleiotropic molecule that as well as binding to FFARs, also has the ability to bind peroxisome proliferator-activated receptors (PPARs) (Are et al., 2008; Alex et al., 2013; Korecka et al., 2013; Mattace Raso et al., 2013; Den Besten et al., 2015; Chitrala et al., 2017). PPARs are a family of ligand-activated transcription factors that recognized to have a significant impact on metabolism related pathways. Due to several regulatory roles in metabolic function and energy hemostasis, they are now one of the most often proposed therapeutic targets for metabolic disorders (Oh et al., 2019). Butyrate could induce adipogenesis by activating PPARγ, which is supported by several studies (Toscani et al., 1990; Li et al., 2014; Yan and Ajuwon, 2015). Adipogenesis is associated with reduced inflammatory and oxidative molecules production in adipose tissue, organs lipotoxicity and IR (Hafidi et al., 2019). In diet-induced obese Apo E−/− mice, oral administration of sodium butyrate (SB) (10 ml/kg diet) increased vascular endothelial growth factor (VEGF) mediated vascularization via upregulating PPARγ contributing to improvement of angiogenesis, inflammation and insulin sensitivity (Aguilar et al., 2018). Anti-angiogenic properties of butyrate have also been proposed both *in vitro* and *in vivo*, which is associated with upregulation of anti-angiogenic VEGF (Ciura and Jagodziński, 2010). Moreover, upregulation of PPARγ by butyrate modulates nuclear factor-κB (NF-κB) pathway, resulting in improvements in insulin signaling and inflammation (Aguilar et al., 2018).

On the other hand, SB may activate AMP kinase (AMPK), which result in upregulation of PPARγ coactivator (PGC)-1α, PPARα and γ (Mattace Raso et al., 2013; Hong et al., 2016). Activation of PPARs induces mitochondria biogenesis through upregulating uncoupling proteins (UCPs) and increases fatty acid oxidation. It is speculated that this is an adiponectin-mediated pathway and recruitment of this pathway starts with upregulation of adiponectin receptors as a result of butyrate supplementation (Hong et al., 2016).

THE EFFECTS OF BUTYRATE ON CVD

Butyrate and Atherosclerosis

An increasing number of studies have found that butyrate can exert protective effects in atherosclerosis. Aguilar et al. first found that supplementation of diet with 1% butyrate could reduce atherosclerotic lesions in ApoE knockout mice by decreasing adhesion molecules production and reducing migration of macrophage to the lesion site (Aguilar et al., 2018). Further *in vivo* research has shown that incubation of macrophage and endothelial cells with butyrate (0.5 mM) for 2 h can increase interleukin-10 (IL-10) production meanwhile can decrease pro-inflammatory cytokines including tumor necrosis factor α (TNFα), IL-1β and IL-6 mainly through suppressing NF-κB pathway. The uptake of oxidized-low density lipoprotein (oxLDL) was also decreased by butyrate treated cells (Aguilar et al., 2018). In Kasahara et al. study, the mice group fed with a 6% tributyrin (TB)-supplemented diet had lower lipid deposition and macrophage accumulation in the lesion. It was also reported that butyrate resulted in improvement of gut permeability (Kasahara et al., 2018).

In high-fat diet-fed ApoE$^{-/-}$ mice, butyrate administration (200 and 400 mg/kg) influenced the microbial composition of the

TABLE 1 | Summary of the studies about the effects of butyrate on atherosclerosis.

Reference	Type of study	Butyrate dose	Model	Result
Aguilar et al. (2018)	*In vivo*	1%wt/wt butyrate in diet for 10 weeks	HFD fed ApoE knockout mice	decreased atherosclerosis lesions of aorta decreased CCL2, VCAM1 increased MMP2 and9 decreased migration of macrophage increased collagen depositions and plaque stability
	In vitro	0.5 mM butyrate for 2 h	Macrophage endothelial cells	decreased ox-LDL uptake, CD36, VCAM1, CCL2, TNFα, IL1β and IL6 increased IL10 levels inhibited NF-kB activity
Kasahara et al. (2018)	*In vivo*	6% wt/wt tributyrin in diet for 14 weeks	ApoE knockout mice	inhibited the development of atherosclerosis, lipid deposition and macrophage accumulation in the plaque reduced gut permeability
Du et al. (2020)	*In vivo*	SB 200 and 400 mg/kg/day for 16 weeks	HFD fed ApoE knockout mice	improved the gut microbial diversity increased the abundance of Firmicutes decreased cholesterol deposition decreased atherosclerotic lesions of aortae decreased TC increased the ABCA1 level in liver
	In vitro	2 and 5 mM butyrate for 24 h	Murine RAW 264.7 macrophages	increased ABCA1 protein level
			ABCA1p-Luc HepG2 cells	increased the cholesterol efflux in RAW 264.7 macrophages in a dose-dependent manner
			Primary peritoneal macrophages	—
Wang et al. (2020)	*In vitro*	100 and 200 μM butyrate for 24 h	TNF-α induced HUVECs cells	decreased VCAM-1 and E-selectin reduced oxidative stress by reducing the levels of ROS and 4-HNE decreased MCP-1 and IL-8 improved protective factor KLF2,via the ERK5 pathway

Abbreviations: HFD, high fat diet; APO E, apolipoprotein E; CCL2, C–C motif chemokine ligand 2; VCAM1, vascular adhesion molecule-1; MMP, matrix metalloproteinases; ox-LDL, oxidized-low density lipoprotein; TNFα, Tumor necrosis factorα; IL, interleukin, NF-kB, Nuclear factor kappa B; SB, sodium butyrate; Hep G2, hepatocye G2; TC, total cholesterol; ABCA1, ATP Binding Cassette Subfamily A Member 1; HUVECs, human umbilical vein endothelial cells; ROS, reactive oxygen species; 4-HNE, 4-Hydroxynonenal; MCP1, monocyte chemoattractant protein-1; KLF2, Kruppel Like Factor 2; ERK5, Extracellular-signal-regulated kinase 5.

gut and improved diversity in favor of increasing Firmicutes specially Bacteroidetes (Du et al., 2020). Additionally, butyrate ameliorated atherosclerosis by downregulating genes involved in lipid metabolism including acyl-CoA thioesterase1 (Acot1), Acot2, Perilipin2 (Plin2), Plin5, Cytochrome4a (10,14 and 31 isoforms) (Du et al., 2020). Results of this study showed that, cytochrome P450 7A1 (CYP7A1) is upregulated in butyrate treated mice and negatively correlated with atherosclerotic lesions (Du et al., 2020). CYP7A1 controls bile acid biosynthesis and helps for the elimination of cholesterol in the liver. This evidence suggests a potential role of butyrate in bile acid metabolism (Li et al., 2013). In the same study, *in vivo*, *ex vivo* and *in vitro* investigations illustrated that butyrate induces ATP-binding cassette subfamily A member 1 (ABCA1) activity in both hepatocytes and macrophages via transcription factor specific protein 1 (Sp1), which causes reduced total cholesterol (TC) and also cholesterol deposition in plaque (Du et al., 2020). ABCA1 is a crucial transporter that contributes to cholesterol efflux toward the biosynthesis of high-density lipoprotein-cholesterol (HDL-C) (Sahoo et al., 2004). Previous studies have shown ABCA1 constrains the formation of foam cells, which are involved in the development of atherosclerosis (Chawla et al., 2001; Joyce et al., 2002).

Inflammation and oxidative stress are well-known in the pathogenesis of atherosclerosis (Ma et al., 2016). In Wang et al. study, butyrate (100 and 200 μM) showed anti-inflammatory and antioxidants effects on TNF-α induced human umbilical vein endothelial cells (HUVECs) by decreasing adhesion molecules (vascular cell adhesion molecule-1 (VCAM-1) and E-selectin) and subsequent THP-1 monocytes attachment, reducing pro-inflammatory cytokines including monocyte chemoattractant protein-1(MCP-1) and IL-8, attenuating oxidants ROS and 4-hydroxy nonenal (4-HNE), improving the protective function of kruple factor 2 (KLF2) via the extracellular-signal-regulated kinase 5 (ERK5) pathway (Wang et al., 2020).

Taken together, these findings indicate that athero-protective effects of butyrate are accompanied by regulating the expression of genes related to lipid and glucose metabolism, improving gut microbiota diversity, suppressing a wide range of inflammatory and oxidative processes, rescuing protective KLF2, and enhancing vascular health. Summary of the studies about the effects of butyrate on atherosclerosis are shown in **Table 1**.

Butyrate and Heart Failure

HF is a condition in which the heart's ability to fill or evacuate blood is compromised (Jin et al., 2020). HF can be caused by any problem that affects the anatomical and/or functional integrity of the heart, such as valve, coronary, or myocardial disease (Luedde et al., 2017).

In a recent study by Mollar et al., butyrate was negatively associated with area under the concentration curve (AUC-H2) in patients with HF, implying that butyrate is lower in patients with higher exhaled hydrogen test (Mollar et al., 2021). Although hydrogen breath tests are not the gold standard for diagnosing small intestinal bacterial overgrowth (SIBO), the results of the aforementioned study suggest that dysbiosis followed by reduced levels of butyrate could play a role in the pathology of HF (Ghoshal, 2011; Mollar et al., 2021). The findings of both *in vivo* and *in vitro* experiments show that butyrate exerts histological cardio-protective effects. Badejogbin et al. examined the impact of SB (200 mg/kg) on heart tissue damage in rats fed either chow or high fat diet (HFD) (Badejogbin et al., 2019). In that research, butyrate significantly ameliorated HFD induced cardio-metabolic abnormalities including hyperlipidemia and glucose dysmetabolism as well as elevated plasma malondialdehyde, corticosterone, and lactate dehydrogenase (Badejogbin et al., 2019). Furthermore, the histological study revealed that butyrate improved cardiac tissue infarction, infiltration, and fibrosis. Butyrate has been shown to protect cardiac tissue architecture and integrity by lowering uric acid (plasma and cardiac tissue) and increasing glutathione antioxidant defenses (Badejogbin et al., 2019).

The incubation of endothelin-1 (ET1) induced cardiomyocytes derived from neonatal rats with butyrate (1–4 mM) inhibited hypertrophic growth of cardiomyocytes by epigenetic gene expression alterations (Umei, 2020). In the same study, transcriptome analysis demonstrated that FFARs didn't have an expression in cardiomyocytes therefore protective anti-hypertrophic action is related to HDAC inhibitory role of butyrate.

In a study conducted by Jiang et al., intraperitoneal butyrate administration (7.5 mg/kg) in rats with myocardial infarction reduced the region of infarction and increased cardiac function by enhancing M2 macrophage polarization, downregulating the expression of inflammatory response-related genes, and suppressing sympathetic nerve remodeling (Jiang et al., 2020). Moreover, SCFAs have been found to influence sympathetic neurons, with the effects mostly relying on the vagus nerve. It seems gut and brain are interconnected mostly via the vagus nerve (Yu et al., 2020).

A growing body of evidence suggests that in addition to butyrate's action on anatomical features of the heart, butyrate may also act through gut-brain neurological processes especially vagal afferent pathway (Li et al., 2018; Onyszkiewicz et al., 2019; Muller et al., 2020; Yu et al., 2020). In this context, li et al. research showed that SB supplementation (5% w/w) improved energy metabolism in HFD fed rats, through the gut-brain neural circuit and these effects diminished after vagotomy (Li et al., 2018). Yu et al. investigated the effect of SB (200 mmol/L) on reperfusion injury in rats underwent vagotomy + myocardial ischemia/reperfusion (I/R) injury or I/R injury alone (Yu et al., 2021). According to previous studies, reperfusion can reduce cardiac function and is associated with an increased risk of HF (Goel et al., 2013). Butyrate significantly reduced infarct size and myocardial damage indicators (plasma lactate dehydrogenase (LDH), creatine kinase (CK), and CK-MB levels). Likewise, butyrate treated rats showed a decrease in I/R-induced oxidative stress, inflammation, and apoptosis. Nonetheless, these effects reversed with a vagotomy (Yu et al., 2021). It is speculated that butyrate improves myocardial I/R injury through the gut-brain neural circuit, and this cardio-protective effect is probably mediated by suppressing sympathetic nervous system. Summary of the studies about the effects of butyrate on HF are shown in **Table 2**.

Butyrate, Hypertension, and Vascular Health

It is hypothesized that butyrate's modulatory effects on BP occur through its interaction with the circulatory system**.** In Sprague-Dawley rats, intramedullary butyrate treatment decreased angiotensin II (Ang II) -induced mean arterial pressure via suppression of (pro) renin receptor (PRR) and its subsequent intrarenal renin-angiotensin system (Wang et al., 2017).

Zhang et al. showed that SB administration (1 g/kg/d) inhibited the activation of the cyclooxygenase-2 (COX-2)/prostaglandin E2 (PGE2) pathway in a HDAC5/HDAC6-dependent manner, contributing to reducing Ang II-induced heart hypertrophy, mean arterial pressure and inflammation (Zhang et al., 2019). Robles-Vera et al. investigated the cardiovascular effects of butyrate (0.5 mg kg day) in spontaneously hypertensive rats (SHR) and control Wistar Kyoto (WKY) rats (Robles-Vera et al., 2020). Butyrate decreased both systolic and diastolic BP and returned T-helper 17 (Th17)/regulatory T cells (Treg) balance in the SHR to WKY rat levels. These effects are mediated by lowering endotoxemia and increasing Treg cells in the vasculature (Robles-Vera et al., 2020). Butyrate in the blood circulation stimulates FFAR3 which is found in veins and contributes to vascular tone. FFAR3 is a hypotensive protein, dilates resistance vessels in an endothelium-dependent manner (Natarajan et al., 2016). Furthermore, FFAR2 and FFAR3 have expression in nerves and evidence revealed their expression is higher in WKY than in SHR rats, therefore, intra brain butyrate administration in WKY had a higher drop in BP than SHR (Toral et al., 2019; Yang et al., 2019). Onyszkiewicz et al. showed that butyrate administration (1.4, 2.8, and 5.8 mmol/kg, intracolonic) caused dose-dependent BP reduction in rats fed a standard diet. It seems these hypotensive effects are mediated via afferent colonic vagus nerve vasorelaxation signaling and FFAR2/3 (Onyszkiewicz et al., 2019).

Nutting and Mortesen studies on arties showed that butyrate causes endothelial-dependent vasodilation in the arteries by increasing cyclic AMP (cAMP) levels (Mortensen et al., 1990; Nutting et al., 1991). In Morikawa et al. study, butyrate administration (1 mM) enhanced nitric oxide (NO) production

TABLE 2 | Summary of the studies about the effects of Butyrate on heart failure.

Reference	Type of study	Butyrate dose	Model	Result
Badejogbin et al. (2019)	*In vivo*	200 mg/kg/day butyrate in diet for 6 weeks	HFD fed wistar rats	ameliorated glucose dysmetabolism decreased TG, TC, corticosterone, MDA, plasma and cardiac UA, and LDH Increased glutathione Reduced cellular infarction, infiltration, and fibrosis
Aguilar et al. (2018)	*In vitro*	1, 2, 4 mM butyrate for 2 h	endothelin-1 (ET1) induced neonatal cardiomyocytes	inhibited hypertrophic growth of cardiomyocytes
Jiang et al. (2020)	*In vivo*	7.5 mg/kg/day butyrate intraperitoneally injected for 3 or 7 days post MI	Sprague-Dawley rats MI model	increased expression of M2 macrophage markers downregulated expression of inflammatory response-related genes suppressed sympathetic nerve remodeling inhibited myocardial hypertrophy
Yu et al. (2021)	*In vivo*	200 mmol/L SB in drinking water for 4 weeks	Sprague-Dawley rats myocardial ischemia/reperfusion (I/R) injury model	decreased infarct size decreased myocardial damage indicators (CK, CK-MB and LDH) decreased inflammation, oxidative stress, and apoptosis suppressed sympathetic nervous system protective effects were diminished by vagotomy

Abbreviations: HFD, high fat diet; TG, Triglycerides; TC, total cholesterol; MDA, malondialdehyde; UA, uric acid; LDH, lactate dehydrogenase; MI, myocardial infarction; SB, sodium butyrate; CK, creatine kinase; CK-MB, creatine kinase myocardial isoenzyme.

TABLE 3 | Summary of the studies about the effects of butyrate on hypertension.

Reference	Type of study	Butyrate dose	Model	Result
Wang et al. (2017)	*In vivo*	1 g/kg/day SB for 14 days	Ang II-infused sprague-Dawley rats model of HTN	decreased Ang II-induced mean arterial pressure decreased gene expression of TNFα and IL6
Zhang et al. (2019)	*In vivo*	1 g/kg/day SB for 2 weeks	Ang II-infused Sprague Dawley rats model of HTN	decreased Ang II-induced mean arterial pressure decreased gene expression of IL-1β, Nlrp3, and MCP-1 in cardiac tissue
	In vitro	2 mmol/L SB	cardiomyocytes H9C2 cells	inhibited cardiac hypertrophy by inhibiting COX2/PGE2 pathway
Onyszkiewicz et al. (2019)	*In vivo*	1.4, 2.8, and 5.8 mmol/kg/day, Intracolonic (IC) or intravenously (IV) butyric acid for 2 days	Wistar rats	IC: increased concentration of butyric acid in the colon, portal and systemic blood, decreased BP and heart rate IV: decreased BP didn't changed heart rate hypotensive effect was depended on vagus nerve signaling and FFAR2/3 receptors
	Ex vivo	5 μM up to 1 mM butyric acid	mesenteric arteries (MA) gracilis muscle arteries (GMA)	butyric acid dilated MA and GMA effective dose was 50 μM up to 1 mM
Robles-Vera et al. (2020)	*In vivo*	0.5 mg/kg/day SB for 13 weeks	WKY and SHR Rats	prevented increase in systolic and diastolic BP prevented increase in Firmicutes/Bacteroidetes (F/B) ratio increased Th17/Treg balance decreased endotoxemia

Abbreviations: SB, sodium butyrate; Ang II, Angiotensin II; TNFα, Tumor necrosis factorα; IL, interlukin; HTN, hypertension Nlrp3; MCP-1, monocyte chemoattractant protein; COX2, cyclooxygenase-2; PGE2, prostaglandin E2; IC, intracolonic; IV, intravenously; BP, blood pressure; FFAR, free fatty acid receptor; MA, mesenteric arteries; GMA, gracilis muscle arteries; WKY, wistar Kyoto rat; SHR, spontaneously hypertensive rat; Th, T helper; Treg, T regulatory.

in interferon treated vascular endothelial cells via increasing expression of inducible NO synthase (iNOS) (Morikawa et al., 2004). It is well known that NO stimulates vasodilation, reduces inflammation, and lowers BP (Napoli et al., 2010). Summary of the studies about the effects of butyrate on hypertension and vascular health are shown in **Table 3**.

BUTYRATE AND CVD RISK FACTORS

Butyrate and Obesity

Several scientific studies confirm obesity as an independent risk factor for CVD, as well as one of the factors increasing the risk of diseases associated with CVD such as dyslipidemia, IR, hypertension, and atherosclerosis (Cercato and Fonseca, 2019). In a recent review by Bridgeman et al., out of 14 studies that examined the effects of butyrate on obesity in HFD fed animals, butyrate significantly was reduced weight gain in 10 studies (Bridgeman et al., 2020). Butyrate exerts its anti-obesity effects mainly through contributing in energy balance equation. First, it reduces calorie intake by reducing appetite and preventing food intake. Second, butyrate increases energy expenditure by affecting metabolic pathways. According to the previous studies, butyrate reduces appetite by increasing anorexic hormones like peptide YY (PYY), glucagon-like peptide 1 (GLP1) through activating FFARs (Lin et al., 2012; Steinert et al., 2017). In a study by li et al., acute and chronic oral butyrate administration (5% (w/w) SB) by attenuating hypothalamic neuronal signaling decreased food intake in HFD mice (Li et al., 2018). However, butyrate had no effect on food intake after vagotomy in mice, suggesting the gut-brain neural circuit involvement. Since it is shown that GLP-1 contributed in the satiety, it may affect the vagal nerve (Li et al., 2018). In addition, butyrate induces the secretion of leptin and adiponectin from adipocytes which are involved in appetite and food intake control (Hong et al., 2016; Yu et al., 2017; Hafidi et al., 2019).

Evidence shows that butyrate increases thermogenesis and promotes fat oxidation by activating brown adipose tissue (BAT), thereby enhancing energy expenditure in the body. Li and hong et al. studies reported that rats consuming butyrate in their diet increased expression of UCPs in BAT and skeletal muscles (Hong et al., 2016; Li et al., 2018). UCPs are mitochondrial proteins and involved in facilitating heat production (thermogenesis) (Ricquier and Bouillaud, 2000). Moreover, the level of tyrosine hydroxylase protein was elevated in BAT by butyrate, which is linked to sympathetic nervous system activity. Therefore, butyrate-induced appetite reduction and BAT activation may depend on gut-brain neural circuitry and vagal nerve signaling (Li et al., 2018).

However, there are no randomized clinical trials that confirm the anti-obesity effect of butyrate and still need to be studied. There is no doubt that weight management strategies can help to combat obesity-related diseases including CVDs (Ebbert et al., 2014). As a result, butyrate supplementation can be considered as an emerging anti-obesogenic agent in the prevention and treatment of obesity and cardio-metabolic disease.

Butyrate and Dyslipidemia

Several studies have demonstrated that butyrate has beneficial effects on dyslipidemia and may be helpful for lowering the risk of CVD, particularly atherosclerosis. We will review the effects of butyrate on triglycerides and cholesterol. The effect of butyrate on triglyceride levels was inconsistent in different studies. Du and Hong et al. reported that butyrate supplementation did not change triglyceride levels (Hong et al., 2016; Du et al., 2020), where as in Li and khan studies butyrate significantly decreased triglyceride levels (Khan and Jena, 2016; Li et al., 2018). Evidence suggests that butyrate has effects on adipogenesis, lipogenesis, and lipolysis (Toscani et al., 1990; Lu et al., 2012; Li et al., 2014; Rumberger et al., 2014; Yan and Ajuwon, 2015; Yu et al., 2017). The positive effect of butyrate on adipogenesis has been reported to be more pronounced. As previously discussed, butyrate promotes adipogenesis by activating the PPAR pathway, thereby reducing circulating fatty acids and their accumulation in vital organs (Hafidi et al., 2019). Furthermore, in Aguilar et al. study, remodeling and proliferation markers such as matrix metalloproteinases (MMP2 and MMP9) and proliferating cell nuclear antigen (PCNA) increased following the increase in the expression of PPAR (Aguilar et al., 2018). MMPs and PCNA are required components for adipogenesis (Blaut and Clavel, 2007; Bauters et al., 2015). Hence, butyrate prevents metabolic disorders such as IR, dyslipidemia, and fatty liver by increasing adipogenesis.

The effect of butyrate on lipogenesis and lipolysis is contradictory and seems to be due to differences in the dose, duration of butyrate treatment, and cell phenotype in the *in vitro* studies. Therefore, since butyrate increases lipolysis in a number of studies and inhibits lipolysis in others, the definitive conclusion about the effect of butyrate on lipid metabolism in adipocytes requires further studies.

Butyrate supplementation has been shown to lower total serum cholesterol in a number of animal studies (Gao et al., 2009; Mattace Raso et al., 2013; Khan and Jena, 2016; Mollica et al., 2017; Zhao et al., 2017; Hu et al., 2018) Cholesterol in the body has two sources, dietary cholesterol and endogenous cholesterol (Kapourchali et al., 2016). According to studies, butyrate can affect both pathways of endogenous cholesterol biosynthesis and dietary cholesterol uptake. Butyrate treatment (200 and 400 mg kg) in HFD fed mice caused reduction in non-high-density lipoprotein cholesterol (non-HDL-C), low-density lipoprotein cholesterol (LDL-C), and total cholesterol (TC) (Du et al., 2020). It is noteworthy that regression analyze revealed that non-HDL-C, LDL-C and TC were positively correlated with percentage of aortic lesions, suggesting that butyrate by down-regulating fatty acid synthesis genes, modifies serum lipid levels and inhibits the progression of atherosclerosis (Du et al., 2020). Alvaro and Marcil reported butyrate decreased 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase gene expression and activity in Caco-2 cells (Marcil et al., 2003; Alvaro et al., 2008). The effect of butyrate on cholesterol biosynthesis is mainly through the downregulation of genes involved in cholesterol synthesis such as isopentenyl diphosphate isomerase, dimethylallyl/geranyl *trans*-

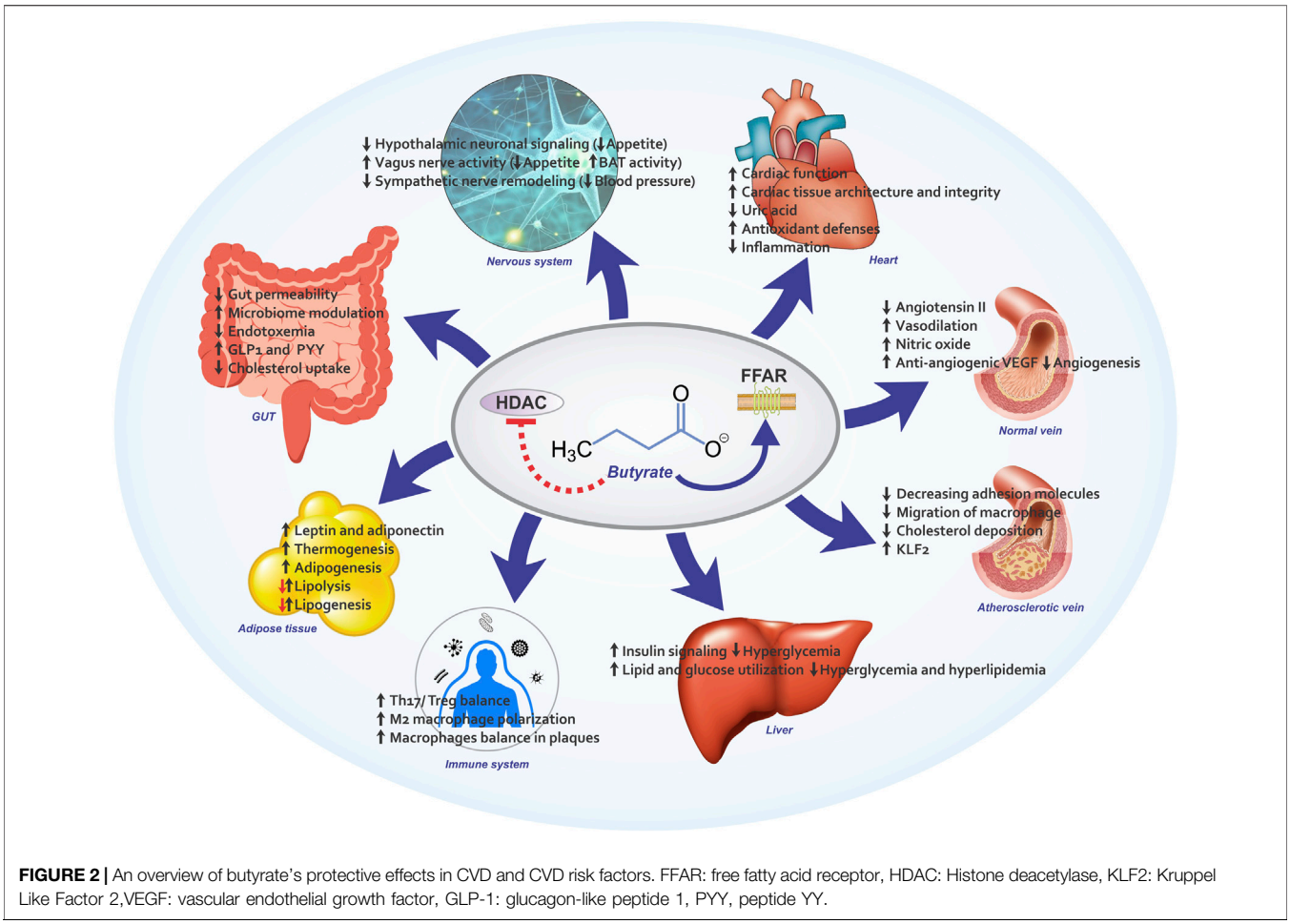

FIGURE 2 | An overview of butyrate's protective effects in CVD and CVD risk factors. FFAR: free fatty acid receptor, HDAC: Histone deacetylase, KLF2: Kruppel Like Factor 2,VEGF: vascular endothelial growth factor, GLP-1: glucagon-like peptide 1, PYY, peptide YY.

transferase and farnesyl-diphosphatase farnesyltransferase. In addition, butyrate prevents intestinal absorption of cholesterol and lowers cholesterol levels. Chen et al. reported that butyrate downregulated Niemann-Pick C1-Like 1 which is involved in intestinal cholesterol uptake (Chen et al., 2018).

Butyrate and Insulin Resistance

There is considerable evidence that both IR and its clinical manifestation, metabolic syndrome, are linked to CVDs (Abdul-Ghani et al., 2019). Numerous studies have shown butyrate can cause an increase in insulin sensitivity by increasing activity of insulin receptors through enhancing insulin receptor substrates. As a result of increased insulin signaling, glucose uptake by cells increases and hyperglycemia alleviates (Yan and Ajuwon, 2015). An increase in glucose transporters (GLUTs) including GLUT2 and GLUT4 after treatment by butyrate has been reported which is further support this hypothesis (Mollica et al., 2017). As mentioned earlier butyrate is also involved in improving insulin sensitivity by increasing adipogenesis (Hafidi et al., 2019). Furthermore, there is evidence indicating that butyrate is effective in reducing hyperglycemia by reducing the expression of gluconeogenesis related genes (Mihaylova et al., 2011; Khan and Jena, 2016). On the other hand, oxidative stress can cause IR with disrupting insulin signaling (Evans et al., 2005). SB administration has been found to reduce oxidative stress in a variety of tissues and cells (Sun et al., 2019). SB induces nuclear factor E2-related factor 2 (Nrf2) and increases expression of downstream antioxidant enzymes, thus contributes in the amelioration of oxidative stress and IR (Sun et al., 2019).

KNOWLEDGE GAPS AND FUTURE DIRECTIONS

Numerous *in vivo* and *in vitro* studies have been conducted exploring the direct and indirect cardiovascular protective capacities of butyrate. The existing body of research on butyrate efficacy suggests that butyrate health promoting effects are mainly due to its two main properties being HDAC inhibitor and activating FFARs. However, in most studies it is not clear exactly, these positive effects are related to which feature. In addition, recent studies have shown new ability such as binding to PPARs that require further study.

However, there is a paucity of clinical trials evaluating efficacy of butyrate supplementation in CVD prevention and treatment. Hence, the main knowledge gap is the lack of human clinical trials to investigate therapeutic benefits of butyrate in CVD. Certainly, large-scale clinical trials with detailed insights of the mechanisms involved are required to confirm the promising effects of butyrate in the management of CVD.

Based on evidence, small amounts of butyrate reaches systemic circulation due to high hepatic clearance (van der Beek et al., 2015). Consequently, placebo-controlled trials of butyrate supplements and butyrate generating bacteria are recommended to determine whether they are effective at increasing systemic butyrate levels. Moreover, Clinical trials are needed to examine the effects of butyrate on AMPK signaling pathway factors like adiponectin receptors, PGC-1α and UCPs which are contributed in energy hemostasis and lipid metabolism.

CONCLUSION

Butyrate has been shown favorable effects in the animal models of CVD as well as CVD-related risk factors such as obesity, dyslipidemia and IR. The beneficial results of butyrate go beyond the gut and it affects various organs as shown in **Figure 2**. Although butyrate exhibits a wide variety of biological activities in different pathways including energy homeostasis, glucose and lipid metabolism, inflammation, oxidative stress, neural signaling, and epigenetic modulation in experimental settings, it remains unclear whether these findings are clinically relevant and whether the molecular pathways are activated by butyrate in humans. Considering several factors that may contribute to cardio-protective activities of butyrate, further well-designed studies are needed to focus on these factors. Understanding exact mechanisms of butyrate in humans will facilitate the application of butyrate as a safe supplement in prevention and management of CVD.

AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows PA and NR: wrote the original manuscript and contributed to the conception of the article; EM, SA, and HT: contributed to data collection and figures designing; SH and ShG: provided advice and consultation; SaG: contributed to the final revision of the manuscript, and all authors: read and approved the final manuscript.

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REVIEW
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Lactoferrin: A Nutraceutical with Activity against Colorectal Cancer

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Homeostasis in the human body results from the tight regulation of several events, since too little inflammation disrupts the process of tissue repair and remodeling, whereas too much exerts a collateral effect by causing tissue damage with life-threatening consequences. In some clinical conditions, such as inflammatory bowel disease (IBD), inflammation functions as a double-edged sword by either enabling or inhibiting cancer development and progression. Generally, cancer develops through evasion mechanisms that regulate cell growth, causing a high rate of uncontrolled proliferation, and mechanisms for evading cell death, such as apoptosis. Moreover, chronic inflammation is a factor that contributes to colorectal cancer (CRC), as observed in individuals with IBD; all these conditions favor an increased rate of angiogenesis and eventual metastasis. Lactoferrin (Lf) is a mammalian iron-binding multifunctional glycoprotein regarded as a natural compound that up- and downregulates both humoral and cellular components of immunity involved in regulating the inflammatory response and maintaining gut homeostasis. Human and bovine Lf share high sequence homology and have very similar antimicrobial, anti-inflammatory, and immunomodulatory activities. Bovine Lf from milk is considered a safe molecule and is commercially available in large quantities. This review mainly focuses on the regulatory effects of orally administered bovine Lf on the inflammatory response associated with CRC; this approach indicates that CRC is one of the most frequently diagnosed cancers and affects the intestinal tract with high clinical and epidemiologic relevance. Thus, this review may provide foundations for the potential use of bovine Lf alone or as a natural adjunct agent to increase the effectiveness and reduce the side effects of anticancer chemotherapy.

Keywords: colorectal cancer, inflammation, lactoferrin, bowel, clinical trials

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1 INTRODUCTION

The aim of this review is mainly to discuss the effects of bovine Lf (bLf) on colorectal cancer (CRC) and the inflammatory response associated with this disease. CRC has high morbidity and mortality rates worldwide. It is the third leading cause of cancer-related deaths, with approximately 0.7 million deaths occurring per year (Kanwar et al., 2015). CRC is a life-threatening cancer affecting the

intestinal tract with clinical and epidemiological impacts (Triantafillidis et al., 2009). Some diseases are associated with a high risk of CRC, such as familial adenomatous polyposis and hereditary nonpolyposis CRC, as well as clinical variants of inflammatory bowel diseases (IBDs), mainly ulcerative colitis and Crohn's disease (Itzkowitz and Yio, 2004; Cutone et al., 2020). CRC has a genetic background in individuals with familial adenomatous polyposis and hereditary nonpolyposis, whereas in patients with IBD, cancer is associated with chronic inflammation (Itzkowitz and Yio, 2004). Patients with IBD are also at risk of suffering small bowel adenocarcinoma and pouch and rectal neoplasms (Triantafillidis et al., 2009). CRC comprises two clinical forms: sporadic CRC and CRC associated with colitis. In sporadic CRC, adenomatous polyps (adenomas) are a major precursor. In CRC associated with colitis, several forms of epithelium dysplasia are observed: polypoid or flat, localized, diffuse or multifocal (Itzkowitz and Yio, 2004). Several factors, such as the interaction with the gut microbiota, genetic background, and immune framework, provide conditions for tumor development that are more frequently found in the colon than in the small intestine (Pancione et al., 2014).

1.1 Colorectal Cancer

All clinical forms of CRC arise from cumulative mutations in cancer regulatory genes or epigenetic alterations in the villi and crypts of the intestinal epithelial cell layer, leading to dysplastic lesions (Simon, 2016). Dysplastic lesions develop slowly from small benign polyps with a low grade of dysplasia and progress to precancerous large polyps with a high degree of dysplasia (low degree of cellular and structural atypia). Enlarged polyps display a greater potential to become localized adenomas that invade the gut wall, and their high rate of neovascularization enables their spread and establishment in lymphatic nodes and adjacent organs, such as the liver, resulting in metastasis with fatal outcomes (Pancione et al., 2014; Simon, 2016). Generally, CRC development results from evasion mechanisms that regulate cell growth, causing a high rate of uncontrolled proliferation. Mechanisms for evading cell death, such as apoptosis and chronic inflammation, are contributing factors to CRC, as observed in patients with IBDs; all these conditions favor an increased rate of angiogenesis and eventual metastasis (Koudougou et al., 2013; Pancione et al., 2014; Jacobs et al., 2015).

Surgery is the first treatment option for nonmetastatic CRC. Patients with advanced CRC additionally require radiotherapy and/or chemotherapy, in which drugs such as 5-fluorouracil (5-FU), oxaliplatin (OXA) (Eloxatin®), and irinotecan (IRI) are used. Furthermore, combined therapy is commonly used. Hand-foot syndrome due to 5-FU or neuro-, oto-, and nephrotoxicity due to OXA are the main unpleasant side effects. Furthermore, a poor response is regularly detected, mainly due to the multidrug resistance of cancer cells. Currently, monoclonal antibodies and multikinase inhibitors have been utilized to treat CRC (Cabeza et al., 2020).

1.2 Intestinal Inflammation and Immune Response in CRC

The development and establishment of CRC evoke the responses of humoral and cell components of host immunity that in turn also participate in the inflammatory response. The interplay between CRC and the immune response may transit through three stages: immune surveillance, equilibrium phase, and immune escape (Koudougou et al., 2013; Jacobs et al., 2015). The immune surveillance phase is accomplished by innate and adaptive immune components that collaborate to eliminate potential tumor cells, mainly if a few cells are present (Koudougou et al., 2013). Immune surveillance encompasses very complex events, including apoptosis of tumor cells, inflammation and the immune response; all these events are mediated by components with an anticancer role in CRC, including innate immune cells such as natural killer cells (NKs), M1-type macrophages, dendritic cells (DCs), mast cells and adaptive immune cells such as TCD8+ cytotoxic cells, TCD4+ helper cells (Th1 phenotype secreting interferon (IFN)-γ, IL-12, and other proteins), Treg cells and Th17 helper cells (Koudougou et al., 2013; Wallace et al., 2014; Jacobs et al., 2015; Abraha and Ketema, 2016). When immune surveillance is surpassed, the equilibrium phase is undetected in the clinic, and the role of the immune system is constrained to limit the growth of tumor cells. The last phase corresponds to immune escape, which is clinically detectable and results from the cumulative evasion of the immune response by tumor cells; this phase arises from immune editing that results from the selection of clones that evade the pressure exerted by the immune system (Koudougou et al., 2013; Pancione et al., 2014; Jacobs et al., 2015).

The intestinal mucosal compartment is the site in which a physiological inflammatory response orchestrated by innate and adaptive mechanisms mediated by intestinal epithelial cells and a wide array of immunocompetent cells in the lamina propria, such as DCs, plays a key role in maintaining gut homeostasis (Kelsall, 2008). The inflammatory response is elicited by germline-encoded pattern-recognized receptors (PRRs) expressed in several cell types that interact with their ligands, namely, pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Some PRRs comprise a large family of receptors, such as Toll-like receptors (TLRs) (Cui et al., 2014).

Upon ligand binding, TLRs trigger signaling pathways, resulting in the activation and translocation of nuclear factor (NF)-κB to the nucleus. NF-κB modulates the expression of proinflammatory cytokines such as interleukin (IL)-1, IL-18, type I interferon (IFN-α and IFN-β), tumor necrosis factor (TNF)-α, and chemoattractant cytokines (chemokines). Another class of PRRs includes NOD-like receptors (NLRs), some of which, such as NLRP1, NLRP3 and NLRP6, function as sensors or adaptors forming "inflammasomes" (Cui et al., 2014). Activation of inflammasomes by PAMPs and/or DAMPs induces signaling pathways and the subsequent activation of caspase-1, which cleaves the inactive pro-forms of cytokines (IL-1 and IL-18) to generate their active forms. Both IL-1β and IL-18 are potent inducers of cancer cell killing by cytotoxic cells (Fabbi et al., 2015). In addition to generating active proinflammatory cytokines, some inflammasomes regulate cell death in response to microbial and

endogenous danger signals (Cui et al., 2014; Parlato and Yeretssian, 2014).

Signaling pathways elicited by PRRs seem to exert a dual effect on cancer cell development. On the one hand, abnormal activation of NF-κB and inflammasomes via TLRs and NLRPs and the resulting cytokine production are the primary causes of chronic inflammation in patients suffering from IBD associated with CRC (Itzkowitz and Yio, 2004; Cui et al., 2014). Loss of control of inflammation, a high rate of cell proliferation, and a low level of apoptotic death exert deleterious effect on gut homeostasis, enabling the formation of conditions for cell transformation and malignancies in the intestine (Itzkowitz and Yio, 2004; Cui et al., 2014; Abraha and Ketema, 2016). On the other hand, PRRs exert an antitumor effect to inhibit tumor progression, showing their dual functions in cancer cells; however, the underlying mechanisms resulting in pro- and anticancer effects have not yet been completely elucidated (Cui et al., 2014). The production of proinflammatory and chemoattractant cytokines exerts a strong effect on the response of components of the innate and adaptive branches of intestinal immunity that accomplishes immune surveillance to sense and eliminate cells with potentially aberrant development (Koudougou et al., 2013; Jacobs et al., 2015). Under normal conditions, physiological inflammation is observed and results from the balance between the pro- and anti-inflammatory interleukin responses from Th1 and Th2 cells located in the intestinal lamina propria (Wallace et al., 2014). Loss of Th1/Th2 regulation may lead to chronic inflammation, which is regarded as a critical factor that favors CRC development; however, a polarized Th1 response may protect against gut dysplasia (Itzkowitz and Yio, 2004; Rizzo et al., 2011). Under conditions of chronic inflammation, IFN-γ secretion by Th1 lymphocytes seems to play a pivotal role in preventing tumor cell proliferation, while IL-13 derived from Th2 lymphocytes and TNF-α, IL-6, and IL-17 produced by Th17 cells promote dysplastic cell proliferation and tumor growth (Rizzo et al., 2011). Interestingly, milk contains bioactive components that promote the Th1 cytokine response while suppressing the Th17 and Th2 responses in the large intestinal lamina propria, as documented in immune milk-fed mice with dextran sulfate-induced colitis (Wang et al., 2014). Thus, humoral and cellular mediators of inflammation and adaptive branches of mucosal immunity seem to function as a double-edged sword in the regulation of cancer cells such that their beneficial effect may be altered by natural immunomodulators present in milk, such as lactoferrin (Lf).

1.3 Lactoferrin Overview

Lactoferrin is a glycoprotein of approximately 80 kDa that belongs to the family of iron transporter proteins (transferrins). Lf differs from other members of the family because it has the highest affinity for iron (Aisen and Leibman, 1972). Lf is a one high cationic polypeptide chain that is highly conserved among mammals (Drago-Serrano et al., 2017). Sorensen and Sorensen identified Lf in bovine milk for the first time in 1939, but it was isolated from human and bovine milk in 1960 (Sorensen and Sorensen, 1940; Groves, 1960; Johansson, 1960). Lf is the second most abundant protein in human milkLf also is present in low quantities in other secretions, such as saliva, bile, semen, tears, and bronchial and intestinal secretions. Lf is part of the secondary granules of neutrophils (Steijns, 2001). In human milk, the Lf concentration (2–4 mg/ml) is higher than that in milk from other species, such as bovine (0.02 and 0.2 mg/ml) or pig, mouse, and horse milk (0.2 and 2 mg/ml). Milk from rats, rabbits, and dogs contains less than 0.05 mg Lf/mL (Masson and Heremans, 1971; Conesa et al., 2008). In humans, the Lf concentration in colostrum is high (6–8 mg/ml), and it has been suggested to provide protection for babies against infectious diseases (Artym and Zimecki, 2005).

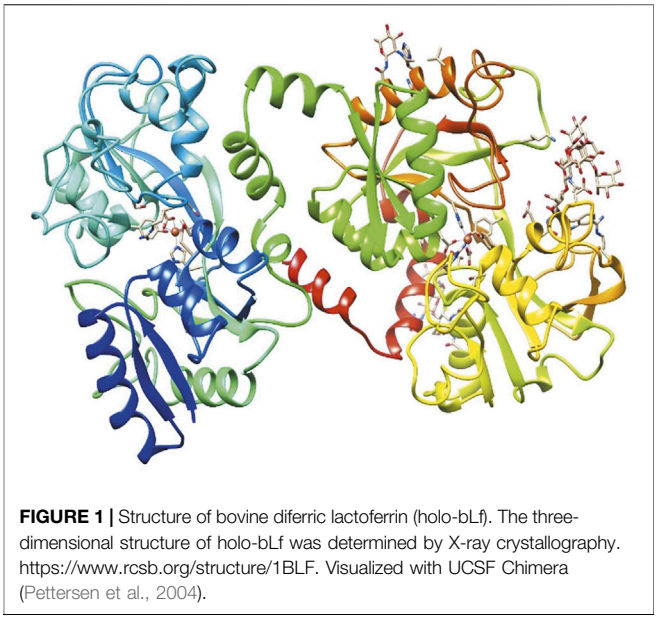

FIGURE 1 | Structure of bovine diferric lactoferrin (holo-bLf). The three-dimensional structure of holo-bLf was determined by X-ray crystallography. https://www.rcsb.org/structure/1BLF. Visualized with UCSF Chimera (Pettersen et al., 2004).

Lf is present in two different forms, depending on its iron chelation state, iron-free (apo-Lf) or bound to two Fe^{3+} iron ions (holo-Lf) (Baker and Baker, 2004). Lf exerts distinct effects on microorganisms in accordance with its iron state. In this sense, holo-Lf may serve as an iron source for some species, while apo-Lf generally causes microbiostasis since it chelates the iron necessary for the growth of pathogens in host fluids and mucous membranes. Apo-Lf also functions as a bactericide that binds and damages the bacterial membrane (Drago-Serrano, 2006). Furthermore, the N-terminus of Lf has been shown to be responsible for the bactericidal effect, since the cleavage of this region by acid-pepsin hydrolysis generates short, active antimicrobial peptides called lactoferricins (Lfcins) and a long peptide sequence called lactoferrampin (Lfampin); in fact, these peptides exert a stronger bactericidal effect than the native molecule (Haney et al., 2012; Zarzosa-Moreno et al., 2020).

Lf is considered a multifunctional protein, because several studies have reported its microbiostatic, microbicidal, anticancer and immunomodulatory properties (Tomita et al., 2009; Roseanu et al., 2010). The molecular mechanisms involve alterations in the virulence factors of microorganisms (Gomez et al., 2003; Ramírez-Rico et al., 2021); binding to host receptors or proteins (Superti et al., 2019; Chang et al., 2020); regulation of

complement (Samuelsen et al., 2004); antioxidant function in oxidative stress; modulation of the cell growth and homeostasis of intestinal microbiota (Baveye et al., 1999; Vega-Bautista et al., 2019); and a positive effect on bone regeneration (Shi et al., 2020) and attenuating obesity (Brimelow et al., 2017). The tertiary structure of holo-Lf is shown in **Figure 1**.

2 LACTOFERRIN AS AN INTESTINAL MODULATOR OF IMMUNITY

Lactoferrin up- or downregulates both humoral and cellular components of immunity. The immunomodulatory effect is derived from its ability to link innate and adaptive immunity to obtain physiological equilibrium. Lf could be considered an alternative molecule for the treatment of diseases that compromise gut immune homeostasis (Legrand, 2016; Drago-Serrano et al., 2017). The balance of homeostasis results from the tight regulation of several events, since too little inflammation disrupts the process of tissue repair and remodeling, whereas too much exerts a collateral effect by causing tissue damage with life-threatening consequences (Luissint et al., 2016). In some clinical conditions, such as IBDs, inflammation functions as a double-edged sword, since it either enables or inhibits cancer development and progression (Rizzo et al., 2011; Formica et al., 2014).

2.1 Modulatory Effects of bLf on the Inflammatory Response in CRC

2.1.1 Physiological Conditions

Bovine Lf exerts modulatory effects on mediators involved in the intestinal inflammatory response, as evidenced in healthy animals such as mice and preterm piglets (Iigo et al., 2004; Spagnuolo et al., 2007; Nguyen et al., 2016). These studies show that the modulation of mediators of intestinal inflammation by bLf depends on the duration of ingestion, dose of bLf and intestinal maturity. For example, an analysis of intestinal lymphocytes isolated from healthy female C57BL/6 mice fed pellets containing bLf for 4 days showed that bLf decreased the expression of the proinflammatory cytokine tumor necrosis factor (TNF)-α and increased the percentage and apoptosis of CD4+ cells among intestinal lymphocytes; however, the percentage and apoptosis of CD8+ cells and caspase-3 and Bcl-2 expression (encoding antiapoptotic protein) in intestinal lymphocytes were unaffected. Based on these results, the decrease in TNF-α levels induced by bLf resulted from increased apoptosis of TNF-α-producing CD4 cells without affecting CD8 cells (Spagnuolo et al., 2007). In contrast, single and/or 7 days of oral administration of bLf or bLfcin increased the activities of the proinflammatory cytokines IL-18 and IFN-γ and caspase-1 in the small intestine of healthy male BALB/c mice; these effects were related to their inhibitory effects on carcinogenesis and metastasis (Iigo et al., 2004).

Moreover, a single administration of bLf by gavage elicited the expression of NOD2-, IFN-β-, and IL-12 in cellular components of innate immunity involved in the inflammatory response in the small intestine of healthy mice (Wakabayashi et al., 2006). The dosage of bLf is critical for exerting beneficial or deleterious effects on intestinal maturation. In preterm piglets, enteral feeding formula supplemented with low doses of bLf increased the maturity and cell proliferation of intestinal cells whereas high bLf doses decreased the villous height and increased the Bax/Bcl-2 ratio (Bax is a proapoptotic protein and Bcl-2 is an antiapoptotic protein) and hypoxia inducible factor (HIF)-1, suggesting increased intestinal apoptosis and inflammation (Nguyen et al., 2016). Chronic HIF-1 expression is a risk factor for intestinal injury and CRC (Shah, 2016). These findings highlight the relevance of the bLf dosage, which provides physiological conditions of inflammatory modulation to be beneficial for gut homeostasis, as described in newborns fed milk containing Lf and other bioactive peptides, such as lysozyme, or in piglets fed recombinant human Lf (rhLf) and/or lysozyme from transgenic cattle and goats (Chatterton et al., 2013; Cooper et al., 2014). In addition, the iron saturation has a critical role on HIF-1 modulation as documented in mice treated human lactoferrin. Unlike the iron saturated counterpart, human apo-lactoferrin acts as a physiological mimetic of hypoxia stabilizes hypoxia-inducible factor-1 alpha as documented in mice (Zakharova et al., 2012).

2.1.2 Inflammation

Lactoferrin exerts a dual effect on the inflammatory response in several models of intestinal cancer that seems to depend on the experimental settings. These models include mice with dextran sulfate sodium (DSS)-induced ulcerative colitis associated with cancer and Apc-Min mice as a model of spontaneous dysplasia (Kanneganti et al., 2011). As described in the murine model of DSS-induced colitis, the cytokine-inflammatory response mediated by IL-1β and/or TNF-α was decreased by hLf and bLf, although a greater beneficial effect was observed for apo-bLf than holo-bLf (Haversen et al., 2003; Li et al., 2013). These findings obtained with bLf are consistent with the anti-inflammatory effect of bovine colostrum by decreasing the expression of the proinflammatory cytokine IL-1β through NF-κB activation in HT-29 cells (An et al., 2009).

On the other hand, bLf also displays proinflammatory activity by enhancing the IL-8 response in Caco-2 cell monolayers stimulated with IFN-γ (Frioni et al., 2014). Moreover, bLf and bLfcin display proinflammatory activities by upregulating cytokines and the activity of cells involved in the inflammatory response to exert antitumor and antimetastatic effects (Wang R. et al., 2000; Shimamura et al., 2004; Kuhara et al., 2006). In mice bearing subcutaneous Co26Lu tumors with high metastatic potential, oral administration of bLf and bLfcin inhibited metastasis, accompanied by increases in the numbers of CD4+, CD8+, asialoGM1+, and IFN-γ-presenting cells and proinflammatory IL-18 and IFN-γ production in the epithelium and/or lamina propria of the small intestine and colon (Wang R. et al., 2000; Kuhara et al., 2006). Bovine Lf increased the expression of proinflammatory cytokines such as type I IFNs, i.e., IFN-α and IFN-β, in Peyer's patches and mesenteric lymph nodes to induce the activation of cytotoxic cells involved in killing tumor cells (Kuhara et al., 2006). The

anticancer effects of bLf were ascribed to its ability to induce the expression of IFN-γ and caspase-1 activation, as found in the murine small intestine. IFN-γ production induced by bLf triggered the cleavage of inactive pro-caspase-1 to generate the active form caspase-1, which in turn cleaved the inactive pro-IL18 to the active IL-18 derivative. Thus, bLf induces IL-18 production through an IFN-γ-dependent mechanism, while bLf induction of IL-18 activation depends on caspase-1 (Iigo et al., 2009). On the other hand, liposomal Lf inhibits the mRNA expression of TNF-α, a proinflammatory cytokine involved in CRC carcinogenesis that is present in human RKO and RCN-9 cells, both of which are CRC cell lines (Sugihara et al., 2017). This presentation of Lf preserves digestion in the stomach and promotes better absorption in the intestinal tract, as described below.

The extent of iron saturation and treatment duration seem to be involved in some antitumor properties of bLf. Experimental assays in tumor-bearing mice showed that unlike the apo form, only fully iron-saturated bLf functioned as an adjuvant for anticancer chemotherapy drugs by increasing Th1 (TNF-α, IFN-γ, and IL-18) and Th2 (IL-4, -5, -6, and -10) responses and nitric oxide (NO) production in homogenates from the small intestine; moreover, the protective effect required bLf feeding for 2 weeks prior to chemotherapy (Kanwar et al., 2008). Unlike Th1 cells, the role of the Th2 response in protection against tumor cells is unclear since it is associated with cancer development (Rizzo et al., 2011; Grizzi et al., 2013; Formica et al., 2014). Moreover, a role of iron in the antitumor action of bLf may be to potentiate the modulatory and effector action of immune cells, as reported in an *in vitro* study in which only iron-loaded Lf stimulated the proliferation and differentiation of cultured lymphoblastic T cells (Bi et al., 1997). Under healthy conditions, iron-unsaturated bLf administered by intragastric intubation or orally also elicited both Th1 (IFN-γ) and Th2 (IL-10) responses in mesenteric nodules and the distal small intestine (Takakura et al., 2006; Arciniega-Martinez et al., 2016) or only a Th1 response, as observed in Peyer's patches isolated from the whole length of the mouse small intestine (Sfeir et al., 2004). Although the role of iron in the antitumor actions of bLf has not been completely elucidated, the reversible iron-binding properties of bLf may be beneficial to balance the iron levels involved in the generation of reactive oxygen species (ROS), causing tissue damage (Kruzel et al., 2007). The modulatory effect of bLf on ROS generation has suggested to protect against inflammation-associated CRC (Fujita et al., 2002; Burrow et al., 2011; Li et al., 2013). Similarly, Burlaka reported that the concentration of Lf varied according to the type of differentiation of neoplasia during CRC metastasis. Lf levels are higher in poorly differentiated neoplasms than in well-differentiated tumors. These results correlated with the iron content in these types of neoplasms, where the iron concentration is four times higher than the value in intestinal mucosa without pathology (Burlaka et al., 2019). Indeed, ROS production during chronic inflammation is associated with the oxidation of DNA, resulting in neoplastic transformation (Itzkowitz and Yio, 2004).

Mechanisms underlying the anticancer activity of bLf are difficult to recapitulate since the multistep character entails the interaction of the immune system with cancer cells, as well as the wide array of bLf properties that modulate innate and adaptive immunity and subsequent inflammation. An overall mechanism of inflammation and its role in dysplastic epithelial cells (**Figure 2A**) entails bLf uptake and translocation through the epithelial layer via receptors, as observed in Caco-2 monolayers (Lonnerdal et al., 2011). After being endocytosed via the intelectin (ITLN-1) receptor, Lf is targeted to the nucleus, where it upregulates IKKα/β expression (Oh et al., 2004; Suzuki et al., 2008). ITLN is a glycoprotein of 105 kDa expressed at the apical membrane of intestinal epithelial cells (Suzuki et al., 2008). Once activated, IKK α/β phosphorylates and concomitantly degrades IκBα, resulting in the release of NFκB (p50/p65) and its translocation to the nucleus to upregulate the expression of the proinflammatory cytokines IL-1β and IL18 (Cui et al., 2014). Although the modulatory effect of bLf on inflammasomes is not known, it is known that bLf translocates to the cytosol where it may activate NOD-like receptor inflammasomes expressed in the intestine (NLCR4 and NLRP6) to regulate the conversion of pro-caspase 1 into active caspase 1 that subsequently cleaves pro-IL18 into the active form IL-18 (Cui et al., 2014).

Receptor-mediated endocytosis of bLf may drive some outcomes (**Figure 2B**). After being endocytosed via the ITLN-1 receptor within epithelial cells, Lf may induce IL-18 production by epithelial cells, which has a pivotal role in the activation of the cytotoxic activity of effector NK and TCD8+ cells (Wang R. et al., 2000; Kuhara et al., 2000). Additionally, the ITLN-1 receptor may facilitate the translocation of Lf to the lamina propria, where it exerts a wide array of modulatory effects; for example, bLf induces macrophage production of IL-18, which is regarded as an anticancer cytokine due to its Th1 polarization activity (Iigo et al., 2009; Fabbi et al., 2015). Lf regulates DCs, which have an essential role as antigen-presenting cells (Puddu et al., 2009). DCs modulate the generation of IL-12 and IFN-γ by Th0 cells to polarize the proinflammatory Th1 response designed to eliminate tumor cells (Kelsall, 2008). Moreover, DCs accomplish cross-presentation through the MHC-I restricted presentation of peptides derived from antigens released by apoptotic cells (for example, epithelial cells) to TCD8+ cytotoxic lymphocytes (Spel et al., 2013). Under chronic inflammatory conditions, IFN-γ secretion by Th1 lymphocytes seems to play a pivotal role in the prevention of tumor cell proliferation (Rizzo et al., 2011); thus, polarization toward a Th1 response by bLf may protect against colon dysplasia.

2.1.3 Cancer

Bovine Lf has been proven to be a prophylactic and chemotherapeutic agent in practically all stages of intestinal cancer including cell proliferation, angiogenesis and metastasis by modulating the innate and adaptive immune responses. Anticancer effects of bLf include its ability: 1) to elicit IL-18 and caspase-1 responses 2) to induce the activation of natural killer cells (NKs), CD4+, CD8+, and IFN-γ T cells, 3) to induce apoptosis of carcinogenic epithelial cells in colon via Fas, caspase-3 and caspase-8 activation and 4) to inhibit angiogenesis (Tsuda et al., 2010). bLf and even its pepsin derivative, hydrolysate

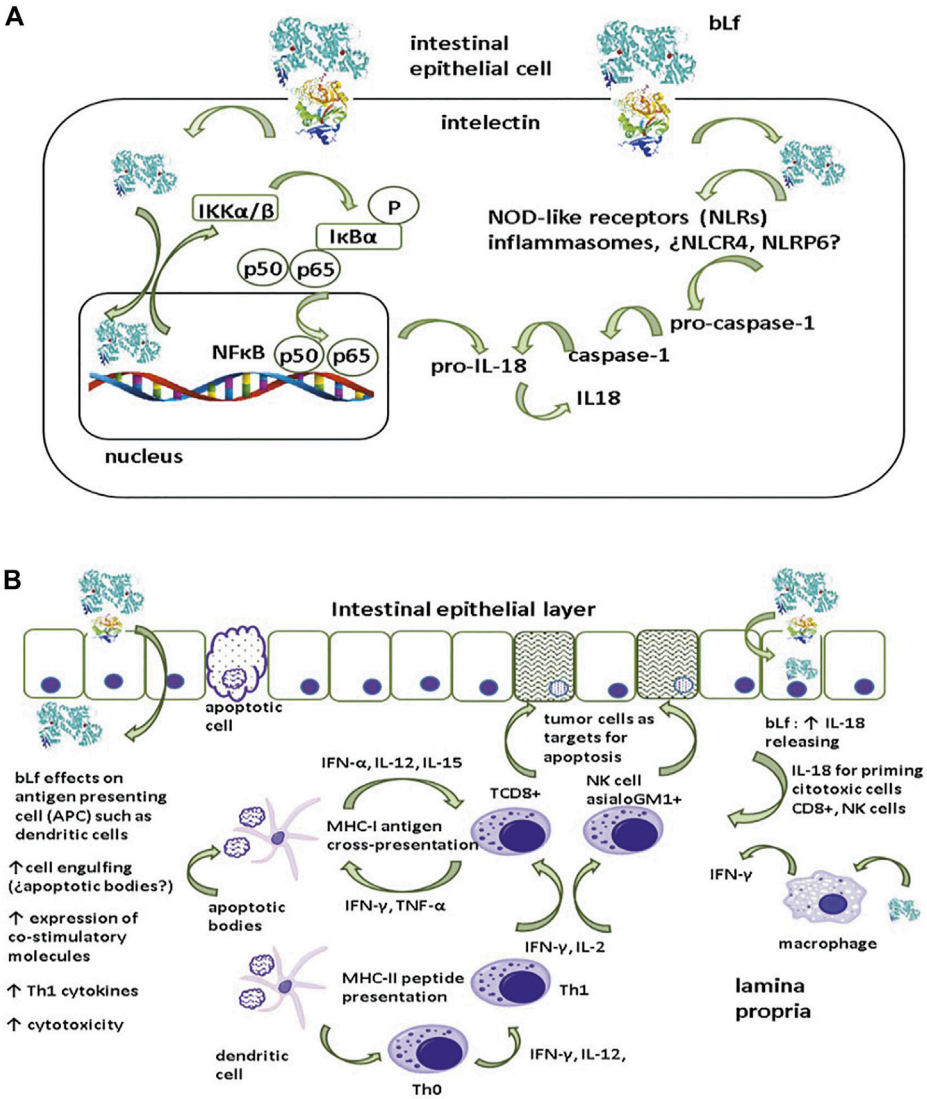

FIGURE 2 | Presumable mechanism of pro-inflammatory cytokine generation by lactoferrin. **(A)** After being endocytosed by intelectin (INTL) by epithelial cells, lactoferrin (Lf) is targeted to nucleus where induces the upmodulation of IKKα/β expression. Once activated, IKK α/β undertakes the phosphorylation and the concomitant degradation of IκBα resulting in the release of NFκB (p50/p65) to be translocated to nucleus to upmodulate the expression of pro-IL18. Additionally, translocation of bLf to cytosol may elicit the activation of Nod-like receptors inflammasomes (¿NLCR4, NLRP6?) involved in the conversion of pro-caspase 1 into the active caspase 1. Caspase 1 accomplishes the conversion of the inactive pro-IL18 into active IL-18. **(B)** After being endocytosed via INTL, Lf induces the production of IL-18 with a pivotal role in the activation of cytotoxic cells essential for the apoptosis of tumor cells. Additionally, INTL receptor may enable the translocation of Lf to lamina propria, where Lf may display a wide array of modulatory actions on macrophages and dendritic cells (DCs). Macrophages are important source of IL-18 and DCs have an essential role as antigen presenting cells that orchestrate the activation of cytotoxic cells and the polarization of pro-inflammatory Th1 response intended for the elimination of tumoral cells via apoptosis (see text for details).

bLfcin, have been tested as chemopreventive, anti-metastatic and anti-tumoral agents in the small and large intestine in experimental assays conducted in rats and mice. These include spontaneous polyp development throughout the intestine in adenomatous polyposis coli (Apc) multiple intestinal neoplasia (Min) mouse (Ushida et al., 1998; Ushida et al., 1999), subcutaneous implantation of highly metastatic colon carcinoma cells in BALB/c mice (Iigo et al., 1999) and azoxymethane (AOM) induced colon tumor in F344 rats (Sekine et al., 1997; Tsuda et al., 1998; Fujita et al., 2002; Ye et al., 2014) (**Table 1**).

In ApcMin mouse model, bLf inhibited the polyposis in the small intestine by decreasing the number of polyps and suppressed polyp generation without displaying toxic effects (Ushida et al., 1998). In BALB/c mice bearing subcutaneous implanted tumors of C26 mouse colon carcinoma (Co 26Lu highly metastatic in lung) both bLf and bLfcin inhibited the lung metastatic colony formation but effects on implanted tumor

TABLE 1 | Trials in humans and assays in animal models with lactoferrin.

Reference	Lactoferrin origin/dose/ duration	Type of tumor/drug	Species	Effect
Studies in animal models				
Sekine et al. (1997)	bLf (0.2 or 2% body weight) 36 weeks	Adenocarcinoma in LI induced by AOM	F344 rats	Significantly reduced with both doses
Tsuda et al. (1998)	bLf (15 mg/kg) 4 or 13 weeks	Adenocarcinoma in LI induced by AOM	F344 rats	Decreased numbers of ACF
	bLf (2, 0.2%, hydrolysate, or 0.1% Lfcin) 36 weeks	Colon adenocarcinomas induced by AOM	F344 rats	Decreased incidences of neoplasia, ACF and β-glucuronidase activity
Ushida et al. (1998)	bLf (0.2 or 2% body weight) 8 weeks	Familial adenomatous polyposis and sporadic colon	ApcMin mouse	Reduction and significant suppression of polyps
	bLf (0.2 or 2% body weight) 8 weeks	CRC induced by AOM	F344 rats	Inhibition of development of ACF
Iigo et al. (1999)	bLf hydrolysate, Lfcin (30, 100, 300 mg/kg) 3–9 or 3–23 days	Implants of the highly metastatic colon carcinoma 26 cells	BALB/c mice	Inhibition of metastasis
Ushida et al. (1999)	bLf (2, 0.2, 0.02 or 0.002% body weight) 8 weeks	Multi-organ carcinogenesis model induced by DEN, DHPN, NMBA	F344 rats	Reduction, suppression and decrease of neoplastic lesions in different organs
Kuhara et al. (2000)	bLf hydrolysate (100 or 300 mg/kg/ day) 1 week	Co26Lu cells were Injected Colon carcinoma on lungs	BALB/c mice	Significant inhibitory effect on metastasis, before and after tumor implantation
Iigo et al. (2009)	bLf hydrolysate, Lfcin, Tf, (30 or 300 mg/kg/day) 7 or 22 days	Co26Lu cells were injected Colon carcinoma on lungs	GKO or BALB/c mice	Inhibition of tumor growth and metastasis
Ye et al. (2014)	18 weeks	CAC induced by AOM and DSS	Lactoferrin knockout mice	Lf was the key in colorectal mucosal immunity and inflammation
Tanaka et al. (2021)	bLf (2% body weight)83 days	Cancer colon induced by AOM and DSS	C57BL/6 J mice	Few lesions in the colon and less weight loss
Clinical trials in humans				
Kozu et al. (2009)	bLf (1.5 g or 3.0 g daily) 12 months	Polyps (adenomas)	Humans (Age 40–75)	Significantly retarded adenomatous polyp growth
Iigo et al. (2014)	bLf (1.5 g or 3.0 g daily) 1 year	Adenomatous colorectal polyps	Humans (Age 63 or younger)	Suppressed growth of colorectal polyps
Moastafa et al. (2014)	bLf (250 mg/day) 3 months	Colorectal cancer	Humans (Age 20–71	Clinically beneficial effect to colorectal cancer patients with better disease prognosis

ACF, aberrant crypt foci; AOM, azoxymethane; CRC, colorectal cancer; DEN, diethylnitrosamine; DHPN, dihydroxy-di-N-propylnitrosamine; NMBA, N-nitrosomethylbenzylamine; DSS, dextran sulfate sodium; CAC, colitis-associated colon cancer.

growth were not seen. In addition, bLf increased the number of asialoGM1+ (natural killer glycolipid marker) and CD8+ cells from peripheral blood. Interestingly, bLf did not exert anti-metastatic activity in athymic nude mice bearing Co 26Lu suggesting that asialoGM1+ and CD8+cells are involved in the anti-metastatic activity of bLf and bLfcin (Iigo et al., 1999). In AOM-induced colon tumors in male F344 rats, bLf and bLfcin had no toxic effects and inhibited the colon tumor and aberrant crypt foci by enhancing the NK cell activity essential for the elimination of tumor cells (Tsuda et al., 1998). In AOM-induced colon tumors in rats was documented that bLf had chemopreventive activity by its apoptotic ability on tumor cells resulting from the increased Fas expression as well the activation of caspase-8 and -3 (Fujita et al., 2004). Most biological functions of bLf including its role on apoptosis may result in part, to the uptake of bLf via receptor by intestinal epithelial cells and its translocation to nucleus as found with human Lf in Caco-2 cell culture assays (Suzuki et al., 2008) (**Table 1**).

Presumable mechanism of apoptosis in tumor epithelial cells (**Figure 3**) involves the interaction of bLf with the ITLN receptor, and once internalized, bLf may be translocated to nucleus where acts as *trans*-activator of p53 promotor via NF-κB activation as described for human neutrophil Lf in HeLa cells (Oh et al., 2004). Lf enhances the activation of IKKα/β kinase resulting in the phosphorylation and degradation of IκBα subunit (Oh et al., 2004); the latter, leads to the release and concomitant activation of NFκB and its translocation to nucleus where induces the transcription of p53 gene in response to DNA damage may lead in cell death (Fulda and Debatin, 2006; Cui et al., 2014). p53 gene protein product induces the expression of pro-apoptotic proteins like Bax that along with Bak are pore forming proteins at mitochondrial outer membrane that permit the outcome of cytochrome C from the interspace membrane to cytosol (Fulda and Debatin, 2006; Chan and Housseau, 2008). In addition, p53 protein attenuates the expression of anti-apoptotic Bcl-2 protein in stem cells located at base of crypts in colon but absent in the murine small intestine; the role of Bcl-2 in attenuation of apoptosis in stem cells may lead to neoplastic transformation frequently found in the large than small intestine (Merritt et al., 1995).

The role of bLf on extrinsic and intrinsic apoptosis pathways is unknown but seem to underlie its anti-tumoral effects as supported in several studies (Tsuda et al., 1998; Fujita et al., 2004). Assays in Lf-knockout mice treated with AOM showed that these animals were highly prone to AOM inflammation-induced colorectal dysplasia that in turn was associated with decreased NF-κB factor signaling and modulation of apoptosis and cell growing (Ye et al., 2014). Fas (also known as CD95 or APO-1) is a transmembrane

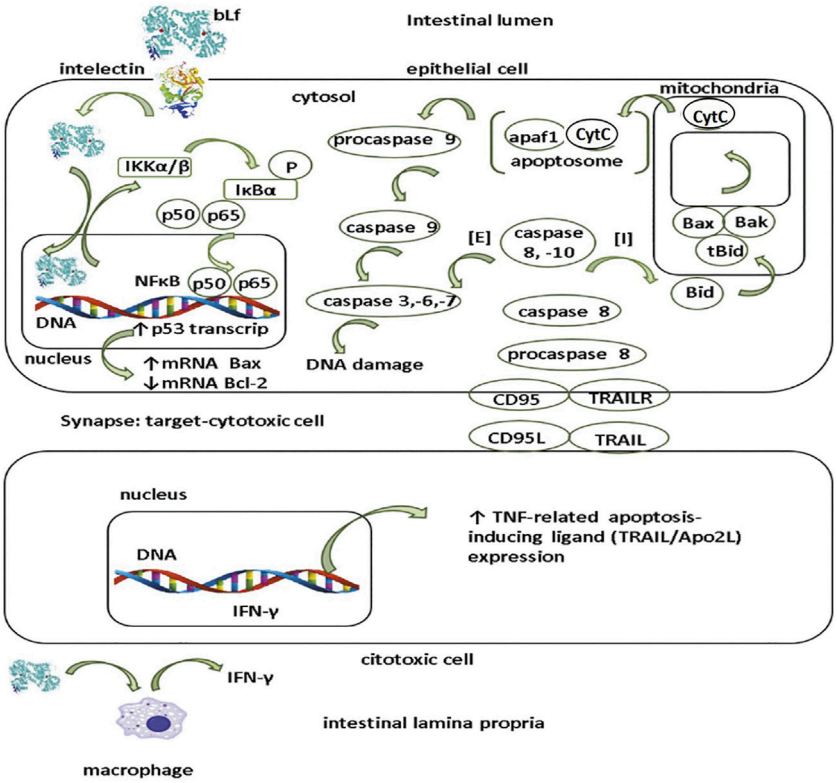

FIGURE 3 | Presumable mechanism of apoptosis by lactoferrin in intestinal tumor cells. Apoptosis pathway may be elicited by lactoferrin (Lf) after being internalized by intelectin (INTL) receptor expressed by intestinal epithelial cells. After translocation, Lf is targeted to nucleus where functions as *trans*-activator of p53 promotor via NFκB promotor activation. Lf induces the IKKα/β activation and concomitant phosphorylation and degradation of IκBα resulting in the release of NFκB (p50/p65) to nucleus. Activation of p53 gene induces the expression of pro-apoptotic proteins (Bax,Bak) while decreases the expression of anti-apoptotic proteins (Bcl-2). Extrinsic (E) apoptosis pathway relies on the ligation of surface molecules on target cells CD95 (Fas/Apo1) and/or TRAILR with their corresponding ligands on the cytotoxic cells CD95L and/or TRAIL respectively. Interaction ligand-receptor enables the conversion of inactive procaspase 8 in the active form as caspase 8. Caspase 8 forms a complex with caspase 10 that triggers sequentially the cascade of activation of caspase -3,-6 and -7 resulting in DNA fragmentation. Intrinsic (I) pathway collaborates in the apoptosis of tumor cells. In this route, caspase 8 and -10 split Bid into the active form tBid (truncated Bid) which in turn activates Bax and Bak. Both Bax and Bak facilitate the outcome of cytochrome C (cytC) from the mitochondrial intermembrane space to the cytosol. Once translocated to cytosol, cytC together with apaf1, form a protein complex called as apoptosome that activates procaspase 9 into caspase 9 that in turn elicits the activation of caspase -3, -6 and -7 resulting in DNA damage. Additionally, at subepithelial level (lamina propria), Lf may enhance apoptosis of tumor cells by eliciting the IFN-γ in macrophages that upmodulates the expression of TRAIL in cytotoxic cells.

protein which belongs to the TNF receptor family endowed with conserved death domains in the intracellular region. After interacting with the ligands (Fas ligand (FasL) and TNF) the conserved death domains of Fas recruit adaptor molecules that elicit signal pathways resulting in the activation the caspase-8 and caspase-3 which are intracellular signaling components from Fas leading DNA fragmentation and ultimately death cell (Fulda and Debatin, 2006; Chan and Housseau, 2008).

Extrinsic apoptosis pathway relies on the ligation of surface molecules on target (epithelial) cells CD95 (Fas/Apo1) and/or TRAILR with their corresponding ligands on the cytotoxic (NK and TCD8+) cells CD95L and/or TRAIL respectively (**Figure 3**). Interaction ligand-receptor enables the conversion of inactive procaspase 8 in the active form as caspase 8. Caspase 8 forms a complex with caspase 10 that triggers sequentially the cascade of activation of caspase -3, -6 and -7 resulting in DNA fragmentation (**Figure 3**). Along with the extrinsic via, the intrinsic (I) pathway collaborates in the apoptosis of tumor cells. In this route, caspase 8 and -10 split Bid into the active form tBid (truncated Bid) which in turn activates Bax and Bak. Both Bax and Bak facilitate the outcome of cytochrome C (cytC) from the mitochondrial intermembrane space to the cytosol (**Figure 3**). Once translocated to cytosol, cytC together with apaf1, form a protein complex called as apoptosome that activates procaspase 9 into caspase 9 that in turn elicits the activation of caspase -3, -6 and -7 resulting in DNA damage and cell death (Fulda and Debatin, 2006; Chan and Housseau, 2008). As depicted in **Figure 2**, one presumable role of bLf after being translocated at subepithelial level is the elicitation of IFN-γ by macrophages (Iigo et al., 2009). Along with IL-18, IFN-γ contributes in priming of cytotoxic cells for killing tumor cells and in antiangiogenic mechanisms (Fabbi et al., 2015).

3 LACTOFERRIN AND ITS DERIVED PEPTIDES IN THE PREVENTION AND THERAPY OF CRC

Chemoprevention of cancer using natural and/or synthetic compounds is an approach to reverse, suppress, or delay the appearance of malignant cells (Zamarin and Postow, 2015; Abraha and Ketema, 2016). The development of targeted therapy has modified the treatment of CRC using agents such as bevacizumab and cetuximab that provide opportunities to treat both locally advanced and metastatic CRC. Nonetheless, all drugs used against cancer have limitations; consequently, scientists are engaged in a continuous effort to develop new therapies. Among the natural compounds, bLf exhibits a wide array of prophylactic and therapeutic effects on intestinal cancer cells, as evidenced in the animal models and human trials (Tsuda et al., 2010) described outlined below.

3.1 Bovine Lf Alone or as an Adjunct Agent as a Treatment for CRC: Assays in Animals

Regarding the experimental use of Lf in CRC models, Dr. Tsuda has been a pioneer in this research field since the 1990s. Sekine et al. examined azoxymethane (AOM)-induced tumors in F344 rats, and afterward, bLf was administered via injections (2 or 0.2% body weight) for 36 weeks. The incidence and number of adenocarcinomas in the large intestine were significantly reduced by both doses of bLf compared with the control group, and no toxic effects were noted (Sekine et al., 1997). In 1998, the same group of researchers documented the effect of Lf on this model, but other variables were introduced, such as other bLf-related products, doses and times of treatment. They divided the study into three experiments: in Experiments I and II, they administered bLf for 4 weeks or 13 weeks, respectively, along with two s. c. 15 mg/kg injections of AOM on days 1 and 8. The numbers of aberrant crypt foci (ACFs) were decreased by both treatments. In Experiment III, animals were administered three weekly injections of AOM and then separated into four groups that received 2 or 0.2% bLf, 2% bLf hydrolysate, or 0.1% bovine lactoferricin (bLfcin) for 36 weeks. Again, no toxic effects were noted, and the incidences of colon adenocarcinomas in the groups were 15, 25, 26.3, and 10%, respectively, in contrast to 57.5% in the control group. The number of ACFs was reduced and beta-glucuronidase activity was decreased in the cecal content of animals receiving bLf (Tsuda et al., 1998). β-Glucuronidase is a lysosomal glycosidase enzyme that catalyzes the degradation of the extracellular matrix of cancer and normal cells and the glycosaminoglycans of the cell membrane, which is important for cancer cell proliferation, invasion, and metastasis. In CRC and other types of cancer, the level of β-glucuronidase activity is increased (Anouar et al., 2019). In addition, the increase NK cell activity induced by bLf indicated that its inhibitory effect might have been related to increased cytotoxicity of immune cells (Tsuda et al., 1998). The results of this group of studies reveal that Lf and its peptides are good tools for CRC treatment and could be used successfully in patients.

Then, another model was established to determine the involvement of bLf in spontaneous intestinal polyp development. This model was assessed in mice that develop both familial adenomatous polyposis and sporadic colon cancers. A reduction in the total number of polyps in the small intestine was observed in the bLf-treated animals, along with significant suppression in the jejunum of animals receiving the 2% dose compared with untreated animals. In addition, body growth suppression (due to anemia and/or intussusception of polyps in the intestine) was alleviated, and no toxic effects were observed on the intestinal epithelium. In these experiments, animals were orally administered 0.2 or 2% bLf as a basal diet for 8 weeks (Ushida et al., 1998), similar to the model of male F344 rats that were induced to develop CRC with AOM, to determine the inhibition of the initiation and early-stage development of ACF by bLf. Although the results were not as obvious as those detected in the rat model, the data suggest that bLf may be a chemopreventive agent for intestinal polyposis.

Subsequently, the effects of bLf and the related compounds bLf hydrolysate and bLfcin on tumor growth and metastasis to the lungs were investigated in BALB/c mice bearing s.c. implants of highly metastatic colon carcinoma 26 (Co 26Lu) cells. Animals that were orally administered bLf and the bLf hydrolysate showed significant inhibition of lung metastatic colony formation from implanted tumors without appreciable effects on tumor growth, while bLfcin showed a tendency to inhibit lung metastasis. Nonetheless, in athymic nude mice bearing Co 26Lu tumors, bLf did not exhibit substantial antimetastatic activity; however, it inhibited lung metastatic colony formation, which was associated with an increase in the numbers of AsialoGM1+ and CD8+ cells in the blood. These results are important for the inhibitory effects of bLf on tumor growth and metastasis (Iigo et al., 1999). Using a rat multiorgan carcinogenesis model, the ability of bLf to inhibit tumor metastasis was tested. The model was developed in male F344 rats receiving i.p. injections of diethylnitrosamine (DEN) and dihydroxy-di-N-propylnitrosamine (DHPN) in drinking water and s.c. injections of N-nitrosomethylbenzylamine (NMBA) during the first 8 weeks (DDN treatment). Then, rats were treated with 2, 0.2, 0.02 or 0.002% bLf administered in the basal diet. Histopathological examinations of neoplastic lesions in the main organs, such as the esophagus, showed a tendency toward a reduction in the bLf-treated animals, along with a significant suppression of relatively large-sized papillomas (more than 50 mm^3 vol) by the 0.2% dose (11% of the control). The multiplicity of adenomas and carcinomas in the lungs was also decreased in animals treated with bLf, which exerted chemopreventive effects on the esophagus and lung, in addition to the colon (Ushida et al., 1999).

In another animal model, oral administration of bLf and its hydrolysate at doses of 100 or 300 mg/kg/day for 1 week exerted a significant inhibitory effect on metastasis before and after tumor implantation (colon carcinoma of the lungs). Some specific effectors of cellular immunity were analyzed, and animals treated with bLf and the hydrolysate exhibited increased numbers of CD4+, CD8+, and asialoGM1+ cells in the spleen and peripheral blood, as well as cytotoxic activities against Yac-1 and Co 26 carcinoma. In the small

intestinal epithelium, numbers of CD4+ and CD8+ cells and the production of interleukin-18 (IL-18) were markedly increased. Therefore, the inhibition of metastasis by oral administration of bLf and its pepsin hydrolysate is mediated by increasing IL-18 production in the intestinal epithelium and activating cellular immunity (Kuhara et al., 2000). Increased production of IL-18 and IFN-γ and caspase-1 activation induced by treatment with bLf are important factors contributing to the increase in intestinal mucosal immunity in tumor-bearing mice (Wang W. P. et al., 2000). In addition, bLf increased Fas expression and apoptosis in the colon mucosa of AOM-treated rats, as well as the expression of proapoptotic Bcl-2 family members (Fujita et al., 2004). Thus, bLf activated an effector pathway mediated by IFN-γ, caspase-1, and IL-18. Additionally, ingested bLf activates multiple effector pathways. For example, in GKO mice, while bLF administration did not activate the IFN-γ/caspase-1/IL-18 effector pathway, it inhibited tumor growth and metastasis by activating the IFN-alpha/IL-7 effector pathway (Iigo et al., 2009).

Ye et al. used an Lf knockout mouse model in which the mice are fertile, develop normally, and display no gross morphological abnormalities, and then chemically induced intestinal inflammation in these animals to investigate the roles of Lf in inflammation and cancer development. These mice displayed a greater susceptibility to inflammation-induced colorectal dysplasia, and this characteristic may be related to the inhibition of NF-kB and AKT/mTOR signaling, as well as the regulation of cell apoptosis and proliferation. The protective roles of Lf in colorectal mucosal immunity and inflammation-related malignant transformation, along with a deficiency in some components of the innate immune system, may lead to serious consequences under condition of chronic inflammation (Ye et al., 2014). A very interesting study was performed by Jiang and Lönnerdal using a transcriptome analysis, which indicated that bLf, cyclic LfcinB, and linear LfcinB exerted antitumor activities by differentially activating diverse signaling pathways, including p53, apoptosis, and angiopoietin signaling. *In vitro* studies using human CRC cells (HT-29) confirmed that both bLf and LfcinBs increase the expression of caspase-8, p53, and p21, critical proteins involved in tumor suppression, providing valuable information on the potential clinical applications of bLf and LfcinB in CRC therapy (Jiang and Lonnerdal, 2017).

Recently, a patient with Crohn's disease was reported to have remained in remission for over 7 years while ingesting 1 g of bLf daily. In a placebo-controlled trial, ingestion of bLf inhibited the growth of intestinal polyps. Thus, the effects of bLf were investigated in a model of CRC related to IBD. The mice were divided into four groups: no treatment, treated with bLf, treated with AOM plus DSS, and treated with AOM + DSS + bLf. AOM was used to initiate intestinal cancer, and DSS was used to induce IBD-like inflammation in the intestine of C57BL/6 mice. The animals treated with AOM + DSS + bLF exhibited a better fecal score, fewer lesions in the colon and less weight loss than the mice treated without bLf, but no differences in the tumor burden were observed (Tanaka et al., 2021).

3.2 Administration of Bovine Lf Alone or as an Adjunct Agent in the Treatment of CRC: Clinical Trials

Since Lf is a natural product from the mammalian innate immune system and has been proven to lack toxicity, it has been used in clinical trials involving patients suffering from distinct types of cancer. These trials are always double-blinded, randomized and controlled. Kozu et al. conducted a clinical trial enrolling 104 patients aged 40–75 years with polyps ≤5 mm in diameter (likely to be adenomas). Participants were assigned to receive placebo, 1.5 g, or 3.0 g of bLf daily for 12 months. Adenomatous polyps were monitored using colonoscopy. In patients who ingested 3.0 g of bLf, significantly retarded adenomatous polyp growth was observed, mainly in patients aged 63 years or younger. This result was very promising because the removal of adenomatous colorectal polyps is performed as a preventive measure against CRC development; however, polyps can be ignored, and when detected, polypectomy is not always an option to eradicate a polyp. Therefore, this clinical trial suggests that daily intake of bLf might be a clinically beneficial adjunct treatment to colorectal polyp extraction (Kozu et al., 2009). Additionally, in a clinical trial published by the same group of researchers, participants who ingested bLf presented increased serum hLf levels, a possible increase in systemic NK cell activity, and increased numbers of CD4+ and CD161+ cells in the polyps. Taken together, these data suggest that bLf suppresses colorectal polyps by enhancing immune responsiveness, consistent with studies performed in rat and mouse models (Iigo et al., 2014). A clinical trial (ClinicalTrials.gov Identifier: NCT01596634) is being conducted to test whether oral bLf reduces taste disturbances in patients with CRC receiving OXA-based chemotherapy.

Another clinical trial examined two groups of patients with CRC to study the therapeutic benefit of orally administered bLf to people who received 5-FU and leucovorin calcium. The test group orally received 250 mg/day bLf in addition to chemotherapy for 3 months, whereas the control group received chemotherapy alone. Although a significant effect was observed that indicated an improvement in the mean percent change in all parameters 3 months after treatment (serum Lf level, serum glutathione-S-transferase enzyme (GST) activity, IFN-γ level, tumor marker carcinoembryonic antigen (CEA), renal and hepatic function tests, and complete blood counts), no significant difference was observed between the results from patients in the test group and in the control group (Moastafa et al., 2014). The purity and quality of bLf administered, doses, times of treatments, and, in general, an overview of the CRC state in participants must be considered.

Because several animal models of all stages of colon carcinogenesis showed promising effects and because those experiments indicated that oral administration of bLf exerts anticarcinogenic effects on the colon and other organs, the participation of bLf in inhibiting the growth of adenomatous colorectal polyps in human patients was corroborated in two clinical trials. As a nutraceutical, bLf and related products inhibit CRC through the following steps: to stop, control, or suppress processes that permit colon cancer growth; to make cancer cells

more recognizable and therefore more susceptible to destruction by the immune system; enhance the killing power of immune system cells, such as T cells, natural killer cells, and macrophages; and to block or reverse the processes that change a polyp to adenocarcinoma. bLf might enhance the ability of the body to repair or replace normal cells that are damaged or destroyed by other forms of cancer treatment, such as chemotherapy or radiation; and to prevent cancer cells from spreading to other parts of the body and inhibit angiogenesis (**Table 1**).

4 DIFFERENT FORMULATIONS OF LACTOFERRIN WITH POTENTIAL ACTIVITY AGAINST COLORECTAL CANCER

Practically, all anticancer drugs are toxic and lack selectivity since they do not distinguish between cancer and normal tissues, inducing severe systemic cytotoxicity and suppression of the immune system; these problems limit their use as treatments for many clinical conditions (Cutone et al., 2020). As described above, Lf is a natural anticancer glycoprotein from the mammalian immune innate system; in addition, Lf is an immunomodulator, and the expression of LfRs, which are expressed in most cells, is substantially increased in tumor cells (Kondapi, 2020). Lf can be used alone or in combination with anticancer drugs, resulting in an increase in drug efficacy. However, Lf is susceptible to degradation by pepsin in the stomach and by trypsin in the small intestine (Rastogi et al., 2014).

Researchers initially proposed linking or coating Lf with some product to protect it from degradation. For example, Ebrahim et al. purified a biomacromolecular complex with a high molecular weight from bovine colostrum whey that contained Lf (HMW-bLf, 250 kDa). This complex showed higher thermal stability and better resistance to gut enzyme digestion than other forms of bLf monomers. In addition, HMW-bLf displayed strong anticancer properties in terms of cytotoxicity and the inhibition of cell proliferation (Ebrahim et al., 2014). Afterward, numerous advances in the development of Lf nanoparticles (NPs) to deliver this glycoprotein with an intact structure and conformational activity, either alone or together with other drugs, using different methods have been reported, all of which use Lf as the basic product, due to its properties of dissolution, pI, and binding to diverse materials. Takeuchi et al. reported that the enteric coating of bLf nanoformulations increased drug transport to the lymphatic fluid within 3 h after intragastric administration, in addition to protecting against digestion by gastric enzymes (Takeuchi et al., 2006). The utilization of NPs containing Lf against diverse types of cancer cells has been recently reviewed (Kondapi, 2020). To the best of our knowledge, clinical trials with NPs containing Lf coupled to drugs have not been performed in patients suffering CRC. Next, we describe some examples of the use of Lf NPs in experiments with CRC cells and animal models. For an explanation of the methods used to prepare nanoparticles, diverse references must be reviewed elsewhere.

As mentioned above, OXA and 5-FU are two of the main chemotherapeutic drugs used to combat CRC in patients. However, they are toxic to the liver, kidneys and bone marrow. In 2018, Ahmed et al. evaluated the antiproliferative potential of 5-FU/OXA loaded in Lf NPs (lacto-nano-5FU and lacto-nano-oxalo) prepared using the Sol-oil method against the COLO-205 cancer cell line. In addition, the authors also i.v. injected these NPs in Wistar rats, and pharmacokinetic parameters and safety were measured. In addition to Lf NPs containing 5-FU or OXA, DSS was also administered to rats in the drinking water to accelerate the process of AOM-ACF induction. The results clearly showed that the 5-FU- and OXA-loaded NPs exhibited enhanced antiproliferative activity and a lower IC50 in the cells than the free soluble drugs. Furthermore, the NPs gently deliver drugs into colon cancer cells and remain there for a longer time than the soluble drugs. Interestingly, colon histopathology showed that the number of ACF was reduced or became normal after treatment with the nanoformulation compared to the positive controls of free drugs. On the other hand, Lf NPs improved the pharmacokinetic profile and biodistribution of the drugs in healthy rats and showed efficacy and safety in the liver and kidneys, as determined by measuring biochemical parameters, and did not reduce the blood cell count in rats with induced colon cancer.

4.1 Nanoliposomes Containing Lf

Nanoliposomes (NLs) are spherical vesicles whose membranes are composed mainly of one or more bilayers of a phospholipid, such as phosphatidylcholine (PC). NLs have been designed since the 1980s to increase the stability of the encapsulated material and protect it from the surrounding milieu (Chen et al., 2012). In addition, other lipids and polyethylene glycol (PEG) have been used instead of PC. NLs are ideal candidates for drug and Lf delivery since they are transported across cell membranes. When administered intravenously, liposomes naturally accumulate in the organs of the reticuloendothelial system, and they have been studied for drug delivery in cancer therapy (Mozafari, 2005; Mozafari et al., 2008). NLs have been utilized as drug carriers for a variety of substances, such as small-molecule drugs, proteins, nucleotides and plasmids, enhancing their activity by improving their stability and permeability and providing targeting and time release (Ishikado et al., 2005; Abu Lila and Ishida, 2017).

Ma et al. compared the effect of NLs containing Lf with that of natural Lf in a Caco-2 cell culture system. Lf-NL phospholipids were stable when evaluated using fatty acid peroxidation assays. The viability of colon cancer cells treated with Lf-NLs was substantially diminished, as determined by their metabolic activity and tests of cell numbers and proliferation. The effects of Lf-NLs on Caco-2 cells included a decrease in membrane integrity (lactate dehydrogenase leakage assay) and ROS generation. Cancer cells also showed morphological changes indicative of apoptosis after acridine orange/ethidium bromide (AO/EB) double staining. Thus, NLs containing Lf might be more efficient against CRC than natural glycoproteins (Ma et al., 2013).

Sugihara et al. evaluated the anti-inflammatory and antitumor effects of liposomal bLf (LbLf) on F344 rats treated with 1,2-dimethylhydrazine (DMH)/DSS. Bovine Lf was coated in soybean lecithin and exhibited improved stability in the stomach and

increased absorption by the intestinal tract compared to bLf alone. DMH is a carcinogen that is widely used to study CRC since it induces the formation of ACF and is involved in the pathogenesis of this cancer. In the assay, rats were randomly divided into three groups: control (water) and groups treated with 500 or 1,000 mg/kg/day LbLf. The rats were injected with DMH (20 mg/kg) once per week for 8 consecutive weeks after 1 week of drinking water containing 1% DSS. All rats were sacrificed at 25 weeks. The tissues were examined for the presence of ACF and for a histopathological analysis. Additionally, human colon cancer cells were utilized to investigate the effect of LbLf on proliferation and inflammation. Rats from the 500 and 1,000 mg/kg/day LbLf groups showed significantly fewer colon ACF, adenomas, and adenocarcinomas than the rats from the control group. The authors also observed that LbLf inhibits cell growth and TNF-α mRNA expression. Therefore, LbLf affects CRC by suppressing inflammation and cell proliferation in rats. This *in vivo* study allows us to infer the preventive and therapeutic value of liposomal bLf in the treatment of human CRC (Sugihara et al., 2017).

4.2 Chitosan Nanoparticles Loaded or Coated With Lf

Chitosan is an N-deacetylated derivative of chitin that is present naturally and abundantly in crab and shrimp shells. Chitosan is a nontoxic, biocompatible, biodegradable, and adsorptive material. In addition, low-molecular-weight chitosan (LMWC) exerts a cytotoxic effect on oral cancer cells (Wimardhani et al., 2014). Chitosan NPs are a drug carrier with wide development potential and have the advantage of slow/controlled drug release, which improves drug solubility and stability, enhances efficacy, and reduces toxicity. In addition to the properties of chitosan *per se*, chitosan NPs can be modified and are muco-adhesive. Because of their small size, chitosan NPs are capable of passing through biological barriers *in vivo*. Therefore, chitosan NPs are used to improve the stability and efficacy of many drugs, including anticancer compounds (Wang et al., 2011). Abu-Serie et al. developed nanocombinations of Lf coated or loaded with lactoperoxidase (LPO, an enzyme present in milk showing antioxidant activity and the ability to degrade carcinogenic compounds). These LPO-loaded chitosan NPs with Lf exhibited increased stability and activity compared to single (free or nanoformulated) bovine proteins. The coating or loading of LPO-loaded NPs with Lf resulted in the highest synergistic cytotoxic effect on Caco-2 cells and greater selectivity in terms of the apoptosis-mediating anticancer effect than other NPs and the free proteins or 5-FU, without causing toxicity in normal cells. This synergistic increase in the anticancer activity was due to apoptosis, which was confirmed by substantial alterations in cellular morphology, a high percentage of annexin-stained cells and sub-G1 populations and nuclear staining with orange fluorescence in treated cancer cells. Additionally, significant alterations in the expression of well-characterized cellular proliferation and apoptosis markers (NF-κB, Bcl-2, and p53) were detected in NP-treated cancer cells compared to 5-FU-treated cells. Although these NPs have not been assayed in animals, they are promising reagents for the development of treatments for human CRC (Abu-Serie and El-Fakharany, 2017).

In another study, Kanwar et al. validated the efficacy of holo-bLf (Fe-bLf) in CRC by targeting survivin to kill colon cancer stem cells. The authors formulated nanocarriers/nanocapsules (NCs) using a complex preparation with calcium phosphate and chitosan and obtained a nanoformulation with a size of 200–250 nm. Fe-bLf was conjugated to the chitosan nanocores using carbodiimide-succinimide for the cross-linking reactions, and the nanocores were coated with alginate solution and calcium chloride (Kanwar et al., 2015). Survivin is a small protein that promotes cancer cell survival by inducing cell cycle progression and inhibiting cell death, suggesting that it may be a molecular target of cancer therapy (Yamamoto et al., 2008). The authors identified the roles of various miRNAs in absorption of the NCs/iron in various mouse organs and tissues. Interestingly, NCs reduced the viability of Caco-2 cells and cancer stem cell markers in triple-positive CD133, survivin and CD44 cancer stem-like cells. In addition, mice treated with NCs did not develop any tumors in a xenograft colon cancer model. One of the receptors for NC internalization is LfR, and in addition to inhibiting angiogenesis and the expression of stem cell markers, NCs also maintain iron and calcium levels (Kanwar et al., 2015).

Wang et al. performed an experiment in murine colon cancer cells (CT26) and human umbilical vein endothelial cells (HUVECs). In addition, they conducted *in vivo* experiments using the subcutaneous xenograft CRC model by performing s.c. injection of CT26 cells into the backs of Balb/c nude mice. The authors reprogrammed the tumor immune microenvironment (TIME) and metabolism via biomimetic targeted codelivery of the anticancer drugs shikonin (SHK) and JQ1. SHK is a phosphatase inhibitor that interferes with cellular signaling mediated by TNF-α and NF-κB (Kondapi, 2020), and JQ1 is a first-in-class potent and selective inhibitor of the Bromodomain-containing protein 4 (BRD4) signaling pathway; it is widely used for tumor biology studies (Shi et al., 2018). Mannopyranoside-Lf (Man-Lf) NPs were prepared using a green method of thermal denaturation, and anticancer drug-encapsulated Lf NPs were obtained using the same method; these NPs were i.v. injected in the animals. The use of Man-Lf-NPs is based on mannose receptors that are expressed on tumor-associated macrophages (TAMs) and facilitate the localization of the NPs and subsequent inhibition of lactate production (Kondapi, 2020). Interestingly, a confocal laser scanning microscopy examination of tumor sections showed that the Man-Lf NPs significantly increased calreticulin (CRT) expression on the membrane of tumor cells, the CRT protein is upregulated in cancer (Zamanian et al., 2013). In addition, significantly greater accumulation of Man-Lf NPs in tumors was observed than that of Lf NPs (Wang et al., 2011). The results suggest that Man-Lf-NPs could be used to target CRC cells in patients.

4.3 Use of Aptamers and Theranostics in CRC

Aptamers are short single-stranded DNA or RNA molecules that can be isolated from large combinatorial libraries through the

systematic evolution of ligands by exponential enrichment (SELEX) procedure (Ellington and Szostak, 1990; Cui et al., 2016). They recognize target proteins, ranging from small molecules to proteins on whole cells, with high affinity and specificity; aptamers have similar properties to antibodies, although they are nanostructured molecules and easily internalized, in addition to showing thermostability, target adaptability, low immunogenicity, and resistance to denaturation (Zhong et al., 2020). All of these properties of aptamers make them potential diagnostic tools for clinical use, such as diagnosis and drug release. In addition, as cancer cells express various tumor-associated membrane proteins on their surface, aptamers combined with molecules recognizing these proteins can target the drug to the cancer cell (targeted therapy). A disadvantage is that aptamers are cleaved by nucleases, but this limitation is solved by modification of the molecule to protect it from these enzymes. Aptamers can be used in the diagnosis, prognosis, and therapy (theranosis) of cancer. Recently, a new technique of cell-based SELEX has been developed and used in CRC; this technique exhibits higher affinity and specificity than SELEX since the target cells are used to screen aptamers (Cui et al., 2016; Chen et al., 2017; Ahmadyousefi et al., 2019).

The first attempt to combine the anticancer therapeutic effects of bLf with the multimodal imaging efficacy of Fe_3O_4 NPs was reported by Roy et al. This combination (Fe_3O_4-bLf) was encapsulated in alginate-enclosed chitosan-coated calcium phosphate (AEC-CP) nanocarriers targeted with locked nucleic acid-modified aptamers against epithelial cell adhesion molecule (EpCAM) and nucleolin. This nanoformulation was orally administered to mice injected with triple-positive (EpCAM, CD133, and CD44) colon cancer stem cells in a xenograft cancer stem cell mouse model. The authors analyzed an ample list of apoptotic, stem cell and angiogenesis markers, cytokines, and gene expression of signaling molecules important in cancer. Interestingly, complete regression of tumors was observed in 70% of mice fed nontargeted (NT) NCs (control), with 30% mice showing tumor recurrence after 30 days. However, only 10% of mice fed targeted NCs showed tumor recurrence, indicating a significantly higher survival rate (Roy et al., 2015). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that activates the apoptosis pathway by binding to its associated death receptors DR4 and DR5 (Yuan et al., 2018). The second mitochondria-derived activator of caspase (SMAC)/direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (DIABLO) protein is an essential and endogenous antagonist of IAPs (Zhao et al., 2020). The authors found that the anticancer mechanism of the NCs was mediated by TRAIL, Fas, Fas-associated protein with death domain (FADD)-mediated phosphorylation of p53 to induce the activation of second mitochondria-derived activator of caspases (SMAC)/DIABLO (inhibiting survivin) and mitochondrial depolarization, leading to the release of cytochrome C. Apoptosis was induced by the inhibition of the Akt pathway and activation of cytokines released from monocytes/macrophages and DCs (Roy et al., 2015). On the other hand, the recurrence of tumors in NC-fed mice mainly occurred due to activation of alternative pathways, such as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and Wnt signaling, leading to an increase in the expression of survivin and other antiapoptotic proteins. Thus, these NCs might be used to treat CRC in patients, and they have the special ability to target tumors, as observed using near-infrared (NIR) imaging, magnetic resonance imaging (MRI) and computerized tomographic (CT) techniques. In this case, real-time cancer therapeutic imaging leading to targeted colonic adenocarcinoma therapy would be feasible. Furthermore, these NCs maintained the immunomodulatory properties of bLf (Roy et al., 2015).

On the other hand, Kamaluparam et al. modified the preparation of NCs to combine the nanotheranostic approach of FebLf NCs and their innate anticancer activity with live *in vivo* imaging using near infrared fluorescence (NIRF) real-time live mouse imaging technology. The authors orally administered NCs to CIMP1+/CIMP2−/CIN + colonic adenocarcinoma tumor-bearing C57 Balb C nu/nu-nude mice. The NCs exhibited great *in vivo* antitumor effectiveness, leading to a reversion of xenograft tumor growth over 90 days, which was the experimental period. NIRF real-time imaging revealed the selective localization of the NCs at the tumor site and subsequent inhibition of tumor growth. *Ex vivo* NIRF imaging of mouse organs showed increased tumor uptake and biodistribution in vital organs, including the spleen, intestine, and kidney, and the histopathological analysis revealed the lack of toxicity of NCs toward mouse tissues. These results confirmed the biocompatible, multimodal anticancer activity of these novel FebLf NCs for real-time cancer therapeutic imaging, leading to targeted colonic adenocarcinoma therapy. Thus, FebLf NCs could be employed to treat human CRC in the near future (Kamalapuram et al., 2016). A review focused on the application of lactoferrin-loaded aptamers and anticancer drug delivery to solid tumors, specifically CRC that addressed the different targeted anticancer approaches was published by Chaudhary et al. (Chaudhary et al., 2017).

5 CONCLUSION AND PERSPECTIVES

The intestinal inflammatory response is an orderly, controlled and organized process that facilitates tissue proliferation and repair. However, chronic inflammation may cause the loss of cellular homeostasis and trigger cellular alterations, including carcinogenesis. Bovine Lf and its peptides, either used alone or as adjuvants, are molecules that exert beneficial effects on inflammation and neoplastic cell proliferation in CRC, participating directly in carcinogenesis or in the modulation of the immune response to this cellular process. The dosage of administered bLf alone or in combination with chemotherapeutic drugs should be accurately measured to provide benefits while avoiding potential risks related to a proinflammatory environment that promotes tumor cell development in the intestine. Trials examining patients with CRC have documented the efficacy of orally administered bLf in the therapy course. Novel nanoformulations of bLf have been investigated in animal models with promising results. In addition, Bovine Lf can be used as a biomarker and a noninvasive diagnostic supporting test in patients with inflammatory disease or infectious bowel disease. Currently human recombinant lactoferrin (Kruzel

et al., 2013) may provide advantages given its compatibility to be used for future in clinical trials to test its anticancer activity.

AUTHOR CONTRIBUTIONS

DGM and D-SME contributed to the idea for the manuscript. R-RG, DGM, L-SN and DGM developed the design and structure of the review. All authors contributed to manuscript revision, read, and approved the submitted version.

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Nutraceuticals in the Modulation of the Intestinal Microbiota: Current Status and Future Directions

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Pharmaceutical interest in the human intestinal microbiota has increased considerably, because of the increasing number of studies linking the human intestinal microbial ecology to an increasing number of non-communicable diseases. Many efforts at modulating the gut microbiota have been made using probiotics, prebiotics and recently postbiotics. However, there are other, still little-explored opportunities from a pharmaceutical point of view, which appear promising to obtain modifications of the microbiota structure and functions. This review summarizes all *in vitro, in vivo* and clinical studies demonstrating the possibility to positively modulate the intestinal microbiota by using probiotics, prebiotics, postbiotics, essential oils, fungus and officinal plants. For the future, clinical studies investigating the ability to impact the intestinal microbiota especially by using fungus, officinal and aromatic plants or their extracts are required. This knowledge could lead to effective microbiome modulations that might support the pharmacological therapy of most non-communicable diseases in a near future.

Keywords: microbiota, immunomodulation, probiotics, bacteria, prebiotic, postbiotic, essential oil, phytotherapy

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INTRODUCTION

Gut Microorganisms and Human Health: A Complex Network

The gut microbiota (GM), i.e., the complex microbial community housed in our gastrointestinal tract, is undoubtedly a leading player in human physiology. Over the years, in fact, it has been shown to be responsible for numerous functions, from the barrier effect to the regulation of metabolism, to the modulation of the immune system, as well as the central nervous system (Sharon et al., 2016; Zheng et al., 2020; Barone et al., 2021). Among others, GM can indeed affect our energy balance and nutritional status, synthesizing essential vitamins (mainly those of group B) and making it possible to digest fibers by fermenting them into short-chain fatty acids (SCFAs, mainly acetate, propionate ad butyrate) (Flint et al., 2015). The latter are microbial metabolites considered essential for the maintenance of our metabolic, immunological, and neurological homeostasis, being involved among others in energy storage and expenditure, appetite control, strengthening of the integrity of the epithelial barrier, induction of interleukin (IL)10 and IL-18 production, and modulation of the synthesis and release of neuroactive substances (Koh et al., 2016; Makki et al., 2018). On the other hand, through the metabolism of dietary components, GM can also generate molecules with known harmful effects on human health, as exemplified by branched-chain fatty acids (related to insulin

resistance, diabetes and inflammation), phenolic compounds (generally linked to poor cardiovascular outcomes), and trimethylamine (converted by the host into the proatherogenic trimethylamine-N-oxide) (see Turroni et al., 2018 for a review on the bioactive small molecules produced and/or contributed by GM and their effects along "the diet from the microbiome to the host axis").

The vast majority of the aforementioned activities are attributed to the bacterial counterpart, by far the most studied and represented within the GM (with estimated over 10 trillion bacteria harboring a genetic potential hundreds of times greater than that of humans) (Sender et al., 2016), although more and more evidence is available on other GM fractions, such as the fungal (mycobiota) and the viral (virome) (Richard and Sokol, 2019; Shkoporov et al., 2019). In this regard, it should be remembered that microorganisms interact with each other in complex (often interkingdom) networks (Santus et al., 2021), whose ecological rules, in terms of positive (i.e., cooperation, commensalism, and cross-feeding), negative (competition, ammensalism, exploitation, and interference) and asymmetric interactions (exploitation, predation and parasitism) are still far from being understood (see Coyte and Rakoff-Nahoum, 2019 for a comprehensive review of microbe-microbe interactions within GM).

Such a complex microbial community is known to be shaped by a series of endogenous and exogenous variables, such as minimally genetics (Rothschild et al., 2018) and mostly exposome, i.e., "life-course environmental exposures (including lifestyle factors), from the prenatal period onwards" (Wild, 2005). Exposome mainly includes exposures related to personal behavior (diet, physical activity, drugs, etc.), occupational and those related to the built and outdoor environment (Zhang et al., 2019). In particular, as mentioned above, diet is widely recognized as one of the main variation drivers of GM, capable of influencing its composition and functionality, and cascading human physiology (Zmora et al., 2019). More recently, however, some authors have introduced the term "geographic effect" to describe the cumulative importance of personal and environmental exposures in driving the GM structure (He et al., 2018).

Based on the above, it is therefore not surprising that GM imbalances (i.e., dysbiosis) have been associated with a number of intestinal and extra-intestinal disorders, including metabolic, hepatic, immunological, respiratory, cardiovascular, neurological, psychiatric and oncological (Lynch and Pedersen, 2016). As will be detailed in the next paragraphs, the alterations in GM are generally featured by a reduction in diversity (a hallmark of health), the enrichment of opportunistic pathogens or pathobionts, and/or the depletion of beneficial microbes, primarily SCFA producers (Duvallet et al., 2017). This arrangement probably compromises the integrity of the epithelial barrier (i.e., leaky gut), with consequent translocation of microorganisms and elicitation of inflammatory states, both locally and systemically, potentially contributing to the onset of a plethora of non-communicable chronic diseases, including autoimmune ones (Gopalakrishnan et al., 2018; Camilleri, 2019). As recently discussed (Sonnenburg and Sonnenburg, 2019a,b), these maladaptive responses of GM are likely the result of a series of Westernization-related factors, including the consumption of industrialized and processed foods with low amounts of Microbiota-Accessible Carbohydrates (i.e., dietary fiber), the routine use of antibiotics and the increased sanitation, which have gradually depleted GM, depriving it of evolutionarily important microorganisms and interactions, thus leading to the establishment of the so-called microbiota insufficiency syndrome (Sonnenburg ED. and Sonnenburg JL., 2019).

Dysbiosis and Non-communicable Diseases

The role of gut dysbiosis in the development of non-communicable disease has been summarized in **Figure 1**.

Dysbiosis and Autoimmune Disorders

Gut Associated Lymphoid Tissue (GALT) represents the immune system component in the gut and it is structured with specific subsets of immune cell populations, such as T and B lymphocytes, which protect the body from intracellular parasites and from perpetuating autoimmune responses (T helper 1 cells, Th1) or provide humoral response to fight extracellular organisms (T helper 2 cells, Th2). A balanced immune response involves the activity of other immune cell subsets such as T regulatory cells (Treg), which limit immune response preventing autoimmune responses, and T helper 17 (Th17), which inhibit Treg proliferation enhancing inflammatory response (Vangoitsenhoven and Cresci, 2020). Autoimmune Diseases (ADs) are disorders characterized by an immune response directed against self-antigenic proteins, which causes damage and loss of function of involved tissues. They are mainly classified as polygenic disorders (rheumatoid arthritis, systemic lupus erythematosus, type I diabetes) when several genes are involved in their pathogenesis, or monogenic disorders, when a single gene is associated with their onset (Liu et al., 2021). Although the presence of a genetic background predisposing to monogenic or polygenic ADs has been discovered, it is widely accepted that gut microbes and their metabolites may play a pivotal role in their onset and maintenance since they may affect intestinal permeability, with bacterial proteins translocating in the bloodstream, which in some cases mimic autoantigens and could interfere in the Th17/Treg cell balance (Atarashi et al., 2011). For example, segmented filamentous bacteria (Sczesnak et al., 2011; Goto et al., 2014) and bifidobacteria (Tanabe, 2013; Tan et al., 2016) can induce Th17 cell differentiation, while *Bacteroides fragilis* (Round et al., 2010), *Lactobacillus reuteri* and *Clostridium* cluster IV, XIVa and XVIII (Atarashi et al., 2011) have been demonstrated to promote the induction of colonic Tregs, the latter with a mechanism involving the production of SCFAs (Pryde et al., 2002). Type 1 Diabetes (T1D) has been associated with severe dysbiosis and increased intestinal permeability. In particular, an increase in *Bacteroides* and *Clostridium* and a decrease in bifidobacteria and lactobacilli have been found in these patients (Murri et al., 2013; Mejía-León et al., 2014; Pinto et al., 2017). Interestingly, *Bacteroides* overgrowth has been linked to Treg depletion and epithelial

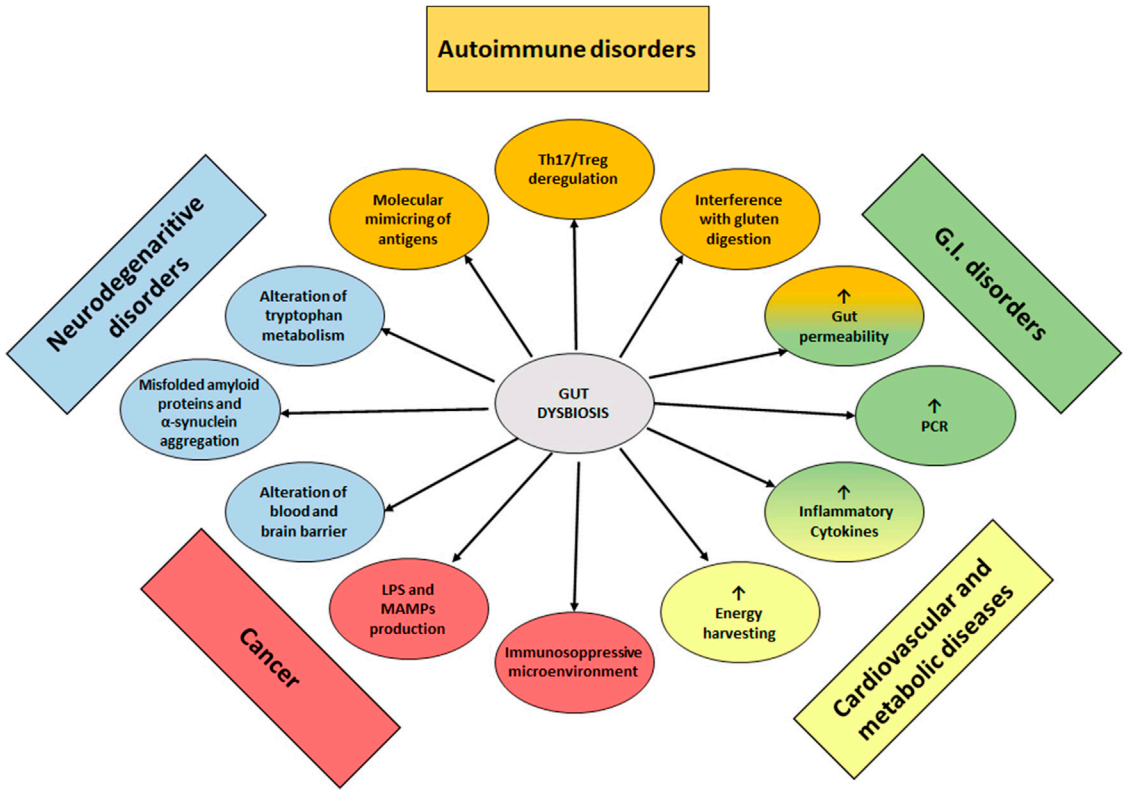

FIGURE 1 | Dysbiosis of GM as a cause or contributing cause of non-communicable diseases.

barrier impairment (Gianchecchi and Fierabracci, 2019). Moreover, in T1D patients, the increase in *Bacteroides* has been correlated to anti-islet cell autoantibodies (Henschel, et al., 2018). Hashimoto's thyroiditis (HT) and Grave's disease (GD) are the two main autoimmune disorders affecting the thyroid and are characterized by the presence of antibodies against thyreoperoxidase, thyroglobulin (in TH) and against thyroid-stimulating hormone (TSH) receptor in GD. In both HT and GD diseases, anti-gliadin, anti-transglutaminase and anti-*Saccharomyces cerevisiae* antibodies have been detected, and both thyroid autoimmune disorders are characterized by intestinal dysbiosis (Köhling et al., 2017). In HT patients, an increase in Prevotellaceae and Pasteurellaceae has been recorded and in particular, at the genus level, an increase in *Blautia*, *Roseburia*, *Ruminococcus*, *Romboutsia*, *Dorea*, *Fusicatenibacter* and *Eubacterium*. On the contrary, a decrease in Enterobacteriaceae, Veillonellaceae and the genera *Rikenella*, *Faecalibacterium, Bacteroides, Prevotella* and *Lachnoclostridium* has been highlighted (Ishaq et al., 2018; Zhao et al., 2018). It is more than plausible that a microbial pattern like this may affect Treg modulation and functions (Köhling et al., 2017). GM can modulate the synthesis of neurotransmitters, such as dopamine, which can inhibit TSH and modulate the hypothalamus-pituitary axis. So, an imbalance in GM could contribute to thyroid disorder development and maintenance with mechanisms involving microbial metabolic pathways (Zhao et al., 2018; Fröhlich and Wahl, 2019). Coeliac disease (CD) is an autoimmune disorder triggered by the interaction between GALT and undigested gluten peptides that translocate across the epithelial barrier into the lamina propria. An increased relative abundance of *Bacteroides*, *Prevotella* and *Escherichia*, and the concomitant reduction of bifidobacteria and lactobacilli have been supposed to contribute to the disease development by influencing the gluten peptide digestion, by stimulating dendritic cells and Tregs, and also by increasing intestinal permeability (Akobeng et al., 2020). Rheumatoid arthritis (RA) onset has been mainly associated with oral dysbiosis and periodontitis (Gianchecchi and Fierabracci, 2019). In the gut of RA patients, an overall decreased microbial diversity has been clearly evidenced (Chen et al., 2016; Picchianti-Diamanti et al., 2018), with a relatively greater abundance of *Prevotella copri* and a decrease in *Bacteroides* (Harmsen and and De Goffau, 2016; Schmidt et al., 2019). Interestingly, a study conducted by Pianta et al. (2017) evidenced epitopes in *P. copri*, which mimic N-acetyl glucosamine-6-sulfatase and filamin, 2 autoantigens highly expressed in RA patient synovia.

Dysbiosis in Gastrointestinal Disorders

Inflammatory Bowel Diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract with unknow etiology. Beyond a widely studied genetic background, lifestyle and diet appear to have a crucial impact on these pathologies. In fact, despite being a typical disease of Western countries, epidemiological studies reveal how their incidence is

increasing in all populations that have adopted a Western lifestyle, characterized by the consumption of a diet enriched in saturated fats, sugars and processed foods. There is growing evidence that chronic inflammation in IBD is sustained by an inadequate response of the immune system to GM leading to unbalanced production of pro-inflammatory cytokines (Rizzello et al., 2019). GM in IBD patients has been extensively analyzed, with results often differing between studies, especially at lower taxonomic levels (Radhakrishnan et al., 2022). Nevertheless, there are some characteristics that have been confirmed by several studies that could explain the aberrant immune response driving chronic inflammation. A decrease in butyrate-producing species *Faecalibacterium prausnitzii* in IBD subjects, compared to healthy controls, has been evidenced and linked to Treg depletion (Sarrabayrouse et al., 2014; Khan et al., 2019; Akobeng et al., 2020). Moreover, an overall decrease in microbial diversity with a reduction of Firmicutes and an increase in Proteobacteria is often found in IBD patients and has been linked, at least in Crohn's disease, to disease severity (Frank et al., 2007; Gevers et al., 2014; Maharshak et al., 2017). It is still debated if the dysbiosis can be linked to IBD onset or if it is a consequence of the disease. However, some clues are provided by studies in IBD patients with anal-ileus pouch. The pouch is a transitional tissue which, after surgery, is colonized *de novo* by GM (Falk et al., 2007; Kohyama et al., 2009). In a prospective study, a decrease in microbial diversity was observed in fecal samples analyzed before surgery in patients developing pouch inflammation, compared to those who maintained a normal (non-inflamed) pouch (Maharshak et al., 2017). Moreover, an increase in *Ruminococcus* and *Clostridium perfringens* was found in a similar study, associated with a reduction in *Blautia* and *Roseburia* (Machiels et al., 2017). The efficacy of antibiotics and some probiotics for the management of inflammatory flares seems to confirm the strong impact that, beyond a well-established genetic background, GM composition and functions may have on these diseases (Dalal et al., 2018). Irritable Bowel Syndrome (IBS) is a functional disorder characterized by abdominal pain, bloating and an altered intestinal habit that defines four main IBS subtypes: diarrhea-predominant (IBS-D), constipation-predominant (IBS-C), mixed diarrhea and constipation (IBS-M) and non-classifiable IBS symptoms (IBS-U) (Hellström and Benno, 2019). GM analysis in these patients revealed a dysbiosis characterized by loss of diversity compared to the healthy population. Again, the results from various studies are often conflicting. For example, the ratio Firmicutes/Bacteroidetes was found to be both higher and lower in different studies (Jalanka-Tuovinen et al., 2014; Pozuelo et al., 2015). Conflicting results were also obtained on the abundance of SCFA producers (Rodiño Janeiro et al., 2018). These differences may be due to the different analytic techniques, but also to the different clinical features that characterize the IBS subtypes and that should be considered in data analysis (Rodiño Janeiro et al., 2018). Overall, the most consistent data in IBS describe increased proportions of Proteobacteria and Firmicutes members, such as *Veillonella* and *Ruminococcus* (Malinen et al., 2005; Tana et al., 2010; Saulnier et al., 2011), and a decrease in *Lactobacillus*, *Bifidobacterium, Faecalibacterium* and methanogens (Malinen et al., 2005; Rajilić-Stojanović et al., 2011; Zhuang et al., 2017; Zhuang et al., 2017).

Dysbiosis in Metabolic and Cardiovascular Diseases

GM regulates the host energy balance by various mechanisms, e.g., by inducing monosaccharide uptake from the gut and hepatic production of triglycerides, by regulating nutrient absorption and the expression of adipokines, such as the fasting-induced adipose factor, which are involved in peripheral fat storage, as clearly shown by studies on germ-free mice (Bäckhed et al., 2004; Tilg et al., 2020). GM is responsible for the production of SCFAs and monosaccharides from indigestible carbohydrates in the diet, such as fiber (Flint et al., 2015). In metabolic disorders such as obesity and type 2 diabetes, which are strictly correlated to cardiovascular outcomes, intestinal dysbiosis triggers or contributes to the exacerbation and chronicization of these conditions by impairing metabolic pathways and inducing chronic low-grade inflammation that is a typical feature of these diseases (Angelakis et al., 2012; Cotillard et al., 2013). Less microbial diversity has been found in obese subjects, with an overrepresentation of proinflammatory taxa, such as *Ruminococcus* and *Bacteroides*, and a decrease in anti-inflammatory species such as *F. prausnitzii* (Cotillard et al., 2013). Moreover, in subjects with metabolic syndrome there is a greater abundance of SCFA producers, with an increased ability to extract calories from low-energy foods (Marzullo et al., 2020). In type 2 diabetes subjects, GM analysis showed a decrease in *Roseburia intestinalis* and *F. prausnitzii*, associated with high intestinal transport (absorption) of sugars (Qin et al., 2012). A common feature in patients with metabolic disorders is the higher proportion of gram-negative bacteria associated with higher levels of lipopolysaccharide (LPS), a bacterial toxin responsible for increased intestinal permeability and endotoxemia, which leads to systemic inflammation mediated by proinflammatory cytokines (Cani et al., 2007; Qin et al., 2012). This feature may be considered the link between metabolic disorders and cardiovascular diseases such as atherosclerosis, which damages blood vessels predisposing to arterial plaque formations (Novakovic et al., 2020). On the other hand, into the atherosclerotic plaques, *Crysenomonas*, *Helicobacter*, *Anaeroglobus, Clostridium, Eubacterium,* and *Roseburia* have been detected (Koren et al., 2011), while in the gut the overrepresentation of *Lactobacillus, Bacteroides, Collinsella,* and *Streptococcus* has been suggested as a diagnostic marker in patients suffering from cardiovascular disease (Emoto et al., 2017). Together with low gut diversity, some bacterial genera such as *Lactobacillus, Oscillibacter, Faecalibacterium* and *Ruminococcus* showed some correlations with a higher level of C-reactive protein, which is considered a marker of inflammation and cardiovascular disease (Kazemian et al., 2020). From the metabolic point of view, some species belonging to Proteobacteria and Firmicutes have been associated with the synthesis of trimethylamine (TMA) and its derivative trimethylamine N-oxide (TMAO), which is considered a risk factor for myocardial infarction and stroke (Tang et al., 2013; Liu et al., 2015; Hu et al., 2017).

Dysbiosis in Cancer

GM involvement in carcinogenesis is mainly linked to the release of some chemical mediators called microorganism-associated molecular patterns (MAMPS), which can enhance tumor progression or cause an impairment of the immune system (Schwabe and Jobin, 2013). In animal models of hepatocellular carcinoma as well as in pancreatic cancer, LPS released from GM is able to activate Toll-like receptor 4 (TLR4) and negatively modulate immune responses (Dapito et al., 2012; Ochi et al., 2012). Furthermore, several studies showed a typical GM signature in cancers, such as colorectal cancer (CRC) in which a particular strain of *Fusobacterium nucleatum* appears to be involved in tumor initiation and progression by inducing the local release of pro-inflammatory cytokines, such as IL-6, IL-8 and TNF-α, thus developing an immunosuppressive tumor-favoring microenvironment (Shang and Liu, 2018). In non-small cell lung cancer, localized depletion of butyrate producers such as *F. prausnitzii, Clostridium leptum*, *Clostridium* cluster I, *Ruminococcus* spp., *Clostridium* cluster XIVa, and *Roseburia* spp., is supposed to deregulate Treg differentiation and immune responses against the cancer cells (Cheng et al., 2019; Gui et al., 2020). Acute lymphoblastic leukemia is characterized by increased abundance of Enterococcaceae, Porphyromonadaceae and other Bacteroidetes members, and by depletion in *Blautia*, Erysipelotrichales, Lachnospiraceae and Clostridiales members in the GM, while in acute myeloid leukemia, an abundance of Staphylococcaceae and Streptococcaceae has been found (Dutta and Lim, 2020).

Dysbiosis and Neurodegeneration

There are different ways by which GM contributes to the physiological modulation of the central nervous system (CNS). For example, commensal bacteria such as *Escherichia* (Lee et al., 2020), *Streptococcus* (Macrì et al., 2018) and *Enterococcus* may contribute to the regulation of dopamine levels, while *Bifidobacterium* and *Lactobacillus* can produce gamma-aminobutyric acid, which is also involved in dendritic and T cell differentiation (Powell et al., 2017). The inflammatory component associated with neurodegeneration and the contribution of GM in the regulation of neurotransmission make it plausible to associate psychiatric or neurodegenerative disorders such as Alzheimer Disease (AD), Parkinson Diseases (PD), Multiple Sclerosis (MS), Amyotrophic lateral sclerosis (ALS) and Huntington's disease with GM alterations (Uniyal et al., 2021). Interestingly, epidemiological data suggest that diet could play a preventive or delaying role in prion and prion-like neurodegenerative diseases, and diet is a major driver of GM variation (Toni et al., 2017). Nevertheless, to date, the most reliable data on the connection between neurodegeneration and GM come from studies on AD and PD. In AD, an altered GM appears to be linked to LPS production, alterations of the blood-brain barrier and deregulation of Treg/Th2 responses, which promote AD development and fibrillogenesis of β-Amiloid (Li et al., 2017; Tsunoda 2017). Instead, amyloid proteins may be released by GM components, and it has been observed that *Escherichia coli*, *Salmonella* Typhimurium*, Bacillus subtilis, Mycobacterium tuberculosis, Salmonella enterica,* and *Staphylococcus aureus* are all bacteria capable of generating functional amyloid, contributing to accumulation of proteinaceous misfolded particles, oligomers and fibrils (Jiang et al., 2017; Fang et al., 2020).

In PD patients, impairment of the motor system has been associated with the presence of non-motor symptoms, linked to autonomic dysfunctions, now recognized as a part of this disease. Among these autonomic dysfunctions, intestinal constipation can arise even 20 years before the onset of motor symptoms and could be linked to GM alterations (Mertsalmi et al., 2017). An increase in the relative abundance of Bifidobacteriaceae, Lactobacillaceae and Verrucomicrobiaceae has been observed (Fang et al., 2020) and the increase in Enterobacteriaceae has been clearly associated with postural instability (Sampson et al., 2016). Furthermore, the decrease in *Prevotella* has been associated with reduced mucin synthesis and increased gut permeability in PD patients (Sampson et al., 2016). These modifications also correlate with alterations in the microbial metabolism of tryptophan and beta-glucuronides (Bedarf et al., 2017), involved in neurotransmitter biosynthesis. In addition to this, GM dysbiosis could be involved in PD development by enhancing alpha-synuclein aggregation in the intestinal submucosa neurons, which may propagate to the CNS via the vagal nerve route (Fang et al., 2020; Uniyal et al., 2021).

The major GM alterations observed in non-communicable diseases are summarized in **Table 1**.

Prebiotics, Probiotics and Postbiotics for the Microbiota Modulation (Effectiveness and Limits)

Due to its crucial role in human pathophysiology, as detailed above, GM is increasingly considered a therapeutic target in multiple clinical settings, and its modulation is strongly believed to represent an important adjunct to current intervention strategies. Historically, such modulation can be achieved through the use of prebiotics or probiotics (or their combination, synbiotics) or, more recently, postbiotics. Below, for each of these manipulation tools, the available evidence and current limitations will be briefly discussed.

Prebiotics, i.e., "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017), typically include dietary fiber and oligosaccharides, such as fructo-oligosaccharides, inulin and galacto-oligosaccharides. Human milk oligosaccharides, conjugated linoleic acids, polyunsaturated fatty acids, phenolics and phytochemicals are still considered candidate, i.e., molecules whose prebiotic potential has been demonstrated *in vitro* or in animal models but for which human evidence is still insufficient. The health effects of prebiotics are innumerable and extend far beyond the gastrointestinal system, including for example immune responses, metabolism, skin, bones, central nervous system, etc. (see Table 1 from Gibson et al., 2017). These effects are mediated by GM fermentation, therefore by the production of metabolites, mainly SCFAs, which derive from the establishment of complex syntrophic networks, mostly involving members of the dominant families Lachnospiraceae, Ruminococcaceae and

TABLE 1 | Main GM modifications (referred to as taxa) observed in several non-communicable diseases.

	Increased taxa	Decreased taxa
Autoimmune disorders		
Type 1 Diabetes	*Bacteroides, Clostridium*	Bifidobacteria, lactobacilli
Hashimoto's Thyroiditis	Prevotellaceae, Pasteurellaceae *Blautia*, *Roseburia*, *Ruminococcus*, *Romboutsia*, *Dorea*, *Fusicatenibacter*, *Eubacterium*	Enterobacteriaceae, Veillonellaceae *Rikenella*, *Faecalibacterium, Bacteroides, Prevotella, Lachnoclostridium*
Coeliac Disease	*Bacteroides*, *Prevotella, Escherichia*	Bifidobacteria, lactobacilli
Rheumatoid Arthritis	*Prevotella copri*	*Bacteroides*
Gastrointestinal Disorders		
Inflammatory Bowel Diseases	Proteobacteria	Firmicutes, *Faecalibacterium prausnitzii*
Pouchitis	*Ruminococcus, Clostridium perfringens*	*Blautia, Roseburia*
Irritable Bowel Syndrome	Proteobacteria, Firmicutes (*Veillonella, Ruminococcus*)	*Lactobacillus*, *Bifidobacterium, Faecalibacterium,* methanogens
Metabolic and Cardiovascular Diseases		
Obesity	*Ruminococcus, Bacteroides*	*F. prausnitzii*
Metabolic Syndrome	*SCFA producers*	
Type 2 Diabetes		*Roseburia intestinalis* and *F. prausnitzii*
Cardiovascular Diseases	*Lactobacillus, Bacteroides, Collinsella, Streptococcus, Lactobacillus, Oscillibacter, Faecalibacterium, Ruminococcus,* TMA producers*	
Cancer		
Colorectal Cancer	*Fusobacterium nucleatum*	
Non-Small Cell Lung Cancer		*F. prausnitzii, Clostridium leptum*, *Clostridium* cluster I, *Ruminococcus* spp., *Clostridium* cluster XIVa, *Roseburia* spp.
Acute Lymphoblastic Leukemia	Enterococcaceae, Porphyromonadaceae	*Blautia*, Erysipelotrichales, Lachnospiraceae, Clostridiales
Myeloid Leukemia	Staphylococcaceae, Streptococcaceae	
Neurodegenerative Disorders		
Alzheimer's Disease	*Escherichia coli**, *Salmonella* Typhimurium*, *Bacillus subtilis**, *Mycobacterium tuberculosis**, *Salmonella enterica**, *Staphylococcus aureus**	
Parkinson's Disease	Bifidobacteriaceae, Lactobacillaceae, Verrucomicrobiaceae, Enterobacteriaceae	*Prevotella*

Bacteroidaceae (Candela et al., 2010). For example, it has recently been shown that inulin-type fructans are not only bifidogenic, but also induce specific changes in human GM, namely increase in bifidobacteria (as primary degraders) and *Alistipes* (which benefits from degradation by *Bifidobacterium* through cross-feeding), and decrease in *Bilophila*, due to reduced pH and altered conjugation ratios of bile acids (Vandeputte et al., 2017). Despite such evidence, it is still hard to say which prebiotic to administer to achieve certain effects in GM, at what dose and whether this dose will actually be tolerable. Some interesting insights in this direction have recently been provided by Deehan et al. (2020). According to the authors, chemically modified resistant starches with small structural differences, i.e., crystalline maize resistant starch and cross-linked tapioca resistant starch, are capable of inducing divergent and highly specific effects on GM, with enrichment in *Eubacterium rectale* or *Parabacteroides distasonis*, that direct changes in the output of butyrate and propionate, respectively. Notably, dominant effects were consistent within treatment groups and dose-dependent with a plateau at 35 g. While it is still impossible to predict with certainty the changes in GM and human physiology, this study opens the door to the fascinating possibility of developing carbohydrates designed *ad hoc* for a targeted, systematic, and precision manipulation of GM and its metabolic functions relevant to health.

Based on consensus panel recommendations in 2014 (Hill et al., 2014), probiotics, *i.e.*, "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host", include microbial species that have been shown in properly controlled studies to confer benefits to health, as well as potential new commensals and consortia, comprising defined strains from human samples, for which adequate evidence of safety and efficacy is available. In contrast, live cultures, traditionally associated with fermented foods and for which there is no evidence of a health benefit, as well as undefined, fecal microbiota transplants must be kept outside the probiotic framework. To date, probiotics are known to exert their health benefits through a series of mechanistic interactions with the host and the GM, concerning the metabolism of nutrients (with improvement of lactose tolerance), the direct and indirect

pathogen antagonism, improved barrier function, immunomodulation, the analgesic effect on visceral pain, the change in signaling to the nervous system and, of course, the alteration of GM (see Suez et al., 2019 for a review on the pros, cons and many unknowns of probiotics). These effects may depend on contact and/or be mediated by surface (e.g., lipoteichoic acid, exopolysaccharides and cell surface appendages) and secreted (e.g., SCFAs, bacteriocins, etc.) molecules. With particular regard to GM, probiotics are able to transiently integrate into GM and, once they become part of the transient microbiota, they can influence its composition and activity in many ways, including stimulation of the resident community by trophic interaction (through metabolites, growth factors, carbohydrate metabolism, mucin degradation), reduction/inhibition of pathogens through alteration of microbial fitness (pH decrease, niche competition, bacteriocins), and indirect impact via host through changes in the intestinal environment (mucin production, increase of sIgA and defensins) (Derrien and van Hylckama Vlieg, 2015). It should be remembered that these mechanisms are not shared by all probiotics known to date and that, in particular, the production of specific bioactives and the immunological, endocrinological and neurological effects tend to be strain-specific (Hill et al., 2014). Furthermore, it must be said that sometimes conflicting data were obtained, partly related to the heterogeneity of probiotic agents, dosage, duration and mode of administration used in the different studies, but also to other host variables that may mask the true probiotic impact, especially the habitual diet. In this regard, fortunately, there is a growing awareness that one size does not fit all but that baseline host and GM features should be taken into account for a precision, personalized therapy (Suez et al., 2019). As a proof of concept, in 2018 some researchers demonstrated that GM profiles may be resistant vs. permissive to probiotics colonization (Zmora et al., 2018), and especially that probiotic interventions, if not tailored, may not only be ineffective but also not entirely risk-free (Suez et al., 2018). Future directions in this field should therefore include changes at multiple levels, such as strain-level resolution of clinical and mechanistic studies, adequate sample size, definition of highly valid and reliable endpoints, reporting of adverse effects, long-term safety assessment and the inclusion of novel candidate microorganisms with suggested health benefits from recent microbiome research, i.e., next-generation probiotics or live biotherapeutics (O'Toole et al., 2017). Regarding the latter, unfortunately most are still at a very early stage of mechanistic investigation, with the exception of *Akkermansia muciniphila*, a mucin degrader proposed and tested for the treatment of obesity and related complications, and recently approved by EFSA in pasteurized form (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) et al., 2021). In particular, in a proof-of-concept exploratory study in overweight and obese human volunteers, pasteurized *A. muciniphila* improved insulin sensitivity and reduced insulinemia, plasma total cholesterol, body weight, fat mass and hip circumference, without affecting the overall structure of the GM (Depommier et al., 2019).

As an alternative to probiotics, with a longer shelf-life and increased safety especially for immunocompromised individuals, the use of non-viable microorganisms and/or their components has been proposed. These are collectively referred to as postbiotics, i.e., "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host", and precisely include non-viable cells with or without metabolites or cell components, capable of conferring beneficial effects to the host directly or indirectly (e.g., enzymes, peptides, teichoic acids, peptidoglycan-derived muropeptides, polysaccharides, cell surface proteins and organic acids) (Salminen et al., 2021). Postbiotics would exert their actions through five postulated mechanisms, namely modulation of GM (e.g., through the antimicrobial activity of bacteriocins or lactic acid, by carrying quorum sensing and quorum quenching molecules, by providing carbon sources and also through competition with pathogens if adhesins remain intact after processing), strengthening of barrier function, modulation of local and systemic immune responses, modulation of systemic metabolic responses, and systemic signaling through the nervous system. Notwithstanding the need for high-quality randomized placebo-controlled (or alternatively, active agent-controlled) trials, the available evidence suggests that postbiotics may prove effective in adults as new antimicrobials, targeted anti-inflammatory and immunoregulatory agents, and signaling molecules, while there is only limited evidence on the health benefits of including them in infant formulas (see Table 2 and 3 from Salminen et al., 2021). Importantly, several issues must be considered for a preparation to be qualified as such, such as detailed description of the starting material (including the molecular characterization of progenitor microorganisms), the means of inactivation (and confirmation that it has occurred) and assurance of safety in the targeted host for the intended use. Only careful control of these parameters will allow reliable and repeatable research for the integrated use of postbiotics in medical and pharmaceutical applications.

Fungus and Plant Extracts for Microbiota Improvement

The modulation of GM and, consequently, of the gut immune system is a key aspect to preserve the correct physiology of the gastrointestinal tract. GM alterations are continuously reflected at the immune level and therefore may become systemic. Consequently, the action of fungi and medicinal plants is exerted on the gastrointestinal system through immunomodulating, antioxidant and protective properties on GM. The protection of the intestinal biofilm and barrier, structures in which the GM actively and directly participates, also fall within the therapeutic actions of fungus and therapeutic plants. These effects on the intestinal barrier and on the gastrointestinal system can have, as we have seen, multiple systemic consequences. Several medicinal plants and fungi are described in the scientific literature as being able to act positively on various acute and chronic inflammatory disorders of the gastrointestinal system, most of these are also part of the medical tradition of one or more regions of the world. Despite this, the actions on the GM have been studied in preclinical and controlled clinical studies for only a few medicinal plants and

fungi. Sometimes, studies in the literature support the possible therapeutic use of some of these fungi and plants only in the modulation of intestinal inflammation. Although it is evident that the inflammatory component and the alteration of the GM are associated in almost all pathologies of the gastrointestinal system, it is not possible to deduce from the effects on inflammation which kind of modulations in the GM actually occurred.

Microbiota-Modulating Fungi

Hericium erinaceus is the most used mushroom for all gastrointestinal disorders. It is an edible mushroom, which has a long history of use in traditional Chinese medicine for the protection of mucous membranes, gastric ulcers, acute and chronic gastritis and nervous degeneration (Khan et al., 2013; Friedman, 2015; Thongbai et al., 2015). The parts of the fungus used are the fruiting body and/or the mycelium in aqueous, hydroalcoholic or alcoholic extracts titrated and standardized in one or more of the following components: polysaccharides, beta-glucans (with antibacterial and anti-inflammatory action), alpha -glucans, diterpenes and triterpenes or polyphenols (He et al., 2017). Although the most studied activities of this fungus concern its immunomodulatory effects on the gut, its prebiotic activities have aroused much interest (Sheng et al., 2017). A single protein, called HEP3, isolated from *H. erinaceus* and administered to rats, treated with trinitrobenzenesulfonic acid (TNBS) to induce experimental colitis, was able to restore microbiota diversity in a relatively short time. Treatment with this single *H. erinaceus* protein increased the amounts of Actinobacteria and Tenericutes, reduced those of Bacteroidetes and Firmicutes, and was able to restore a biodiverse and healthy ecological structure (Diling et al., 2017). The efficacy of HEP3 in positively modulating the GM has also been confirmed in other animal models of colitis (Shao et al., 2019). Crude extracts of *H. erinaceus* were also tested on animal models of colitis. The results indicate that the formulations used (polysaccharide extract, alcoholic extract or whole extract) were able to positively modulate the GM, but while the polysaccharide extract appeared to play an important prebiotic role, the alcoholic extract and the whole extract showed important bactericidal effects (Diling et al., 2017). Similar results were obtained in a mouse model of sodium sulfate dextran-induced colitis (DSS). Treatment with DSS resulted in an increase in the relative abundance of Verrucomicrobia and Actinobacteria and a decrease in the amount of Bacteroidetes in fecal samples, compared to the control group. Treatment of colitic mice with dry extract of the fermented mycelium of *H. erinaceus* reversed most of the changes, including the increased levels of *A. muciniphila*. Taken together, these results showed that *H. erinaceus* effectively modulates the GM of colitic animals, restoring a microbial composition similar to that of healthy rodents (Ren et al., 2018).

Inonotus obliquus commonly known as Chaga is a parasitic fungus mainly of Birch trees (Betulaceae family) with numerous biological properties, and has been commonly used as a folk remedy in Northern European countries for various disorders affecting the digestive system (Shashkina et al., 2006; Balandaykin and Zmitrovich, 2015). The most used formulations are powder, aqueous extract and hydroalcoholic extract, which can be titrated in polysaccharides, beta-glucans, alpha-glucans and polyphenols. *I. obliquus* has also been successfully used to ameliorate the negative effects of DSS in mice, and the polysaccharides of this fungus have shown a positive regulatory effect on the microbiota (Chen et al., 2019). In a model of mice with chronic pancreatitis, the GM profile, compromised by the disease, was partially restored by the administration of *I. obliquus* polysaccharides, which led to increased diversity and richness of GM and also improved the clinical condition of the mouse (Hu et al., 2017). *Ganoderma lucidum* (Reishi in Japanese) is a mushroom with a woody consistency and a bitter taste, which grows preferably on oaks and chestnuts. The main traditional use in China and Japan is aimed at counteracting the allergic and inflammatory state (Bhardwaj et al., 2014). Recent studies have identified more than 400 bioactive molecules present in this mushroom. Some of these were firstly identified in this species and consequently their name referred to the species, such as ganoderiol, ganolucidinic acids and ganoderman-triol (Ahmad, 2018). There is strong evidence of prebiotic activity of *G. lucidum*, although this may be secondary to a direct effect on components of the immune system. In DSS-induced colitis in rats, *G. lucidum* glucans increased SCFA-producing bacteria such as *Ruminococcus*, and reduced pathogens such as *Escherichia* and *Shigella* in both small intestine and cecum (Xie et al., 2019). In high fat diet (HFD)-fed mice, which exhibit body weight gain and diet-associated dysbiosis, treatment with *G. lucidum* mycelium reverted HFD-induced intestinal dysbiosis, decreasing the Firmicutes-Bacteroidetes ratio and the Proteobacteria relative abundance. Furthermore, Reishi treatment reduced metabolic endotoxemia by restoring the integrity of the intestinal barrier. This demonstrated that one of the main mechanisms of action of *G. lucidum* in the intestine is related to the modulation of GM. Polysaccharides with a high molecular weight (>300 kDa) have been identified as the most responsible for this modulation of GM, since these polysaccharides are present in *G. lucidum* in considerable amounts (Chang et al., 2015). Similar results were obtained in a rat model of type 2 diabetes, in which treatment with *G. lucidum* reduced the relative abundance of harmful bacteria, such as *Aerococcus, Ruminococcus, Corynebacterium,* and *Proteus*, while increased that of *Blautia, Dehalobacterium,* and *Parabacteroides*. GM analysis indicated that Reishi treatment could also restore the microbial metabolism of amino acids, carbohydrates, inflammatory substances and nucleic acids, altered by obesity and diabetes (Chen et al., 2020). In a mouse model of pancreatitis induced by diethyldithiocarbamate (DDC), polysaccharides of *G. lucidum* were able to positively modulate the GM, decreasing the relative abundance of Bacteroidetes and increasing that of Firmicutes. Reishi polysaccharide supplementation increased the relative abundance of beneficial bacterial families, such as Lactobacillaceae and Lachnospiraceae, especially *Roseburia*. These results confirmed that the therapeutic mechanism on chronic pancreatitis could also depend on the restoration of a healthy eubiotic GM (Li et al., 2016).

Microbiota-Modulating Plants

It should be noted that all plants, if rich in fiber content, can have prebiotic activities. Despite this, some plants in particular have

shown the ability to modulate the GM in a much more decisive way than would be expected from their unique prebiotic effect due to their fiber content. *Cichorium intybus* is a perennial herbaceous plant whose rhizome and roots are traditionally used in Europe for the treatment of gastrointestinal disorders (Thumann et al., 2019). Used to supplement the diet of farmed broilers and to improve their production performance, *C. intybus* has been shown to induce significant changes in the ileal microbiota, consisting of lowering *E. coli* counts and increasing *Lactobacillus* ones. These effects have been clearly associated with improved growth performance. Dietary *C. intybus* powder has an undoubted prebiotic effect linked to the high content of soluble fiber, and in particular inulin (Khoobani et al., 2019). However, different modulations of the Firmicutes/Bacteroidetes ratio and of some bacterial genera, such as *Alloprevotella, Blautia, Alistipes* and *Oscillibacter*, were observed in mice fed with different chicory cultivars, with a variable effect depending on the genotype of the chicory and not on the fiber content (Khoobani et al., 2019). *Boswellia serrata* is an arboreal plant that forms an aromatic resin also known as "frankincense". *B. serrata* resin was used as a supplement in rabbit diets at different dosages to obtain changes in the caecal microbiota. Results indicated that substantial changes were found in the microbial populations in the cecum of rabbits treated with *B. serrata*, with a significant decrease in total bacterial count and in particular a decrease in *Salmonella enteritidis* and *E. coli* compared to the untreated control group. These results could be attributed to the high polyphenol content of *B. serrata* and to the presence of boswellic acids, which have a powerful antimicrobial effect (Ismail et al., 2019). *Pistacia lentiscus* is a shrub or small evergreen tree that produces a resin called Chios mastic gum, used as a natural food supplement. The effect of *P. lentiscus* was studied in mice with obesity, non-alcoholic steatohepatitis (NASH) and HFD-induced liver fibrosis. Treatment with *P. lentiscus* promoted a partial but significant recovery of GM diversity associated with a decrease in the relative abundance of Bacteroidetes (Kannt et al., 2019). *Olea europaea* is an evergreen fruit tree that is traditionally found in the Mediterranean area. The extra virgin oil (EVO) obtained from the fruits of this plant is able to induce a greater GM biodiversity and promote the growth of beneficial commensal bacteria, both in humans and in laboratory animals (Marcelino et al., 2019). The leaf extract of *O. europaea* administered to obese mice was able to improve their GM by partially restoring the quantities of Actinobacteria, Bacteroidetes and Verrumicrobia. Furthermore, the relative abundance of *Akkermansia* spp. was restored, suggesting a possible positive effect on intestinal barrier functions in treated mice (Vezza et al., 2019). As for *Angelica arcangelica, Achillea millefolia* and *Cetraria islandica*, officinal plants traditionally used to treat intestinal dysbiosis and inflammation, there are no scientific studies published so far to support their positive modulation action on GM. This does not mean that these medicinal plants are not effective in modulating GM ecology, but only that documented scientific evidence of their alleged therapeutic activities is still too scarce.

Essential Oils as Potential Bowel "Eubiotics"

The alteration of the ecologically stable environment of GM can be produced by different causes, such as broad-spectrum antibiotic therapies, xenobiotics in foods, or growth of pathogenic bacterial strains that can interfere in this equilibrium and cause dysbiosis, low-grade gut inflammation or even colitis (Petersen and Round, 2014; Spisni et al., 2020). Thus, GM dysbiosis over time can trigger alterations in the microbial metabolome, increasing the production of toxins that could activate the innate immune response leading to chronic low-grade intestinal inflammation, which could be one of the triggers for the development of several diseases (Kim and Jazwinski, 2018).

Essential oil (EO) molecules are capable of selectively targeting some bacterial species, especially pathobionts, leaving unaltered the bacterial populations considered healthy (Thapa et al., 2012). Furthermore, they are able to counteract the growth of some fungi resident in GM, such as *Candida albicans* whose overgrowth may causes severe opportunistic infections in humans (Saracino et al., 2022). For these reasons these molecules can be considered as eubiotic agents capable of counteracting intestinal dysbiosis. A number of various EO molecules (i.e., eugenol, thymol, piperine) have been used to positively modulate the broiler chicken GM in different studies. The overall results demonstrated that these compounds represent an effective supplementation for the improvement of GM ecology in farmed chickens with a clear eubiotic effect resulting in increased growth performance (Weber et al., 2012).

Geraniol (Ge-OH), an aliphatic terpene alcohol present in EOs extracted from different plants, such as Palmrose (*Cymbopogon martini*), has demonstrated robust anti-dysbiotic activities in mice whose GM ecology was disrupted by DSS administration. Ge-OH, both enema or orally-administered, was able to prevent colitis-associated dysbiosis in treated mice (De Fazio et al., 2016). The eubiotic effect of Ge-OH could be considered multi-target, since this EO compound is a natural inhibitor of the enzyme cyclooxygenase-2 (COX-2), whose activity in the gut wall strongly contributes to intestinal inflammation. Since chronic low-grade inflammation and dysbiosis enter a self-sustaining loop, the multitarget eubiotic action of Ge-OH tends to restore the gut microbial ecology by contrasting both dysbiosis and inflammation. This is probably the reason why the efficacy of Ge-OH delivered directly to the colon reached that of corticosteroid therapy in this model of DSS-induced colitis (De Fazio et al., 2016). In IBS patients, Ge-OH administration led to GM increased biodiversity increased relative abundances of *Collinsella* and especially *Faecalibacterium*, a well-known health-promoting butyrate producer consistently found to be decreased in IBS patients (Rizzello et al., 2018). D-Limonene, administered to HFD mice reduced obesity and reversed many different microbiota signature due to the unbalanced diet, decreasing Peptostreptococcaceae, Desulfovibrionaceae and Erysipelotrichaceae genera in treated mice and increasing the relative abundance of Bacillaceae, Planococcaceae and Clostridiaceae (Valerii et al., 2021). By using an *in vitro*

TABLE 2 | Fungus, plants and EO molecules as GM modulators in clinical studies and in preclinical model of non-communicable diseases.

Diseases/Therapies	Model	Main findings
Gastrointestinal disorders		
Colitis		
Hericium erinaceus (HEP3 protein)	Rats, TNBS; Rats, acetic acid	Overall reversal of colitis-associated dysbiosis (rise of Actinobacteria, Tenericutes, SCFA producers, decrease of Bacteroidetes and Firmicutes)
Hericium erinaceus (dry extract	Mice, DSS	Overall reversal of DSS-induced dysbiosis (decrease of *Akkermansia muciniphila*, increase of SCFA producers)
Ganoderma lucidum (glucans)	Mice, DSS	Overall reversal of DSS-induced dysbiosis (rise of *Ruminococcus*, decrease of *Escherichia* and *Shigella*)
Geraniol	Mice, DSS	Overall reversal of DSS-induced dysbiosis (increase of Lactobacillaceae, Bacillaceae and Bacteroidetes)
IBS		
Geraniol	Human	Overall reversal of IBS-associated dysbiosis (increase of *Collinsella* and *Faecalibacterium*)
Pancreatitis		
Inonotus obliquus (polysaccharides)	Mice, DDC	Overall reversal of DDC-associated dysbiosis (increase of Bacteroidetes, decrease of Firmicutes)
G. lucidum (polysaccharides)	Mice, DDC	Overall reversal of DDC-associated dysbiosis (decrease of Bacteroidetes and increase of Firmicutes, Lactobacillaceae, Lachnospiraceae, *Roseburia*)
Metabolic Disorders		
Obesity		
G. lucidum (mycelium	Mice, HFD	Overall reversal of HFD-induced dysbiosis (decrease of the Firmicutes-Bacteroidetes ratio, and Proteobacteria)
Pistacia lentiscus	Mice, HFD	Reversal of HFD-induced dysbiosis (partial recovery of diversity, decrease of Bacteroidetes)
Olea europaea	Mice, HFD	Reversal of HFD-induced dysbiosis (rise of Actinobacteria, Bacteroidetes, Verrucomicrobia, *Akkermansia* spp.)
D-Limonene	Mice, HFD	Reversal of HFD-induced dysbiosis: increase of Bacillaceae, Planococcaceae, Clostridiaceae; decrease of Peptostreptococcaceae, Desulfovibrionaceae, Erysipelotrichaceae
T2D		
G.lucidum (Glucans)	Rat, HFD and streptozotocin	Overall reversal of T2D-associated dysbiosis (decrease of *Aerococcus, Ruminococcus, Corynebacterium* and *Proteus*, increase of *Blautia, Dehalobacterium, Parabacteroides*)
Disease-Unrelated Eubiotic Properties		
Cichorium intybus	Farmed broilers	Significant changes in the ileal microbiota (lower *Escherichia coli,* rise of *Lactobacillus*)
C. intybus	Mice	Lowering of the Firmicutes/Bacteroidetes ratio, increased *Alloprevotella, decreased Blautia, Alistipes* and *Oscillibacter*
Boswellia serrata (resin)	Rabbit	Decrease of bacterial counts, *Salmonella enteritidis* and *E. coli*
Oregano EO (in combination with trybutirin and methyl salicylate)	Piglets	Increase of Firmicutes, decrease of Proteobacteria, Actinobacillus, *Escherichia*

human colon model, D-Limonene was also capable to selectively reduce *Clostridium* group IV (Nissen et al., 2021).

The use of broad-spectrum antibiotics against infectious diseases or to counteract the overgrowth of pathobionts in various diseases affecting the intestine, such as IBS (Andrews et al., 2021) or IBD (Townsend et al., 2019), could be associated with a transient dysbiosis in the gastrointestinal tract (Çalışkan et al., 2022). Thus, a study evaluated the possibility to substitute antibiotic therapy with a mixture containing EOs was performed on piglets. Dietary inclusion of the combination of tributyrin with methyl salicylate and oregano EO was compared to antibiotic treatment in terms of bowel health. The results demonstrated an improved intestinal morphological structure in weaned piglets, with an improved ratio of villus height to crypt depth in their intestine. Moreover, the results showed major changes in the profiles of GM and bacterial metabolites, which were beneficial to the animal health. In conclusion, this study demonstrated that the combination of Tributyrin with methyl salicylate and oregano EO could be a potential alternative to antibiotics as a feed additive in pigs (Zhang et al., 2020).

In 2009, a preliminary *in vitro* study examined the potential of a selection of EOs as agents to treat dysbiosis. These EOs were examined using the agar dilution method and doubling dilutions against 12 intestinal bacteria species, which represent the major taxa found in the human gastrointestinal tract. The best results were obtained using *Carum carvi, Lavandula angustifolia,*

Trachyspermum copticum, and *Citrus aurantium* var. amara, so these EOs have been found to be the most promising in the treatment of intestinal dysbiosis, even if more research is needed to investigate the tolerability, safety and selective action of these EOs on other bacterial species (Hawrelak et al., 2009).

A recent study was made to investigate the effects of replacing antibiotics with a combination of plant EOs on the growth performance and gastrointestinal health of broilers. Seven hundred and twenty 1-day-old male broilers were randomly divided into 3 experimental groups: the control treatment, the antibiotic supplementation treatment, or the EO supplementation treatment. The EO supplement consisted of a standard combination of ingredients: eucalyptus EO (25%), carvacrol (35%), cinnamaldehyde (25%), capsaicin (10%), and some other prebiotics (5%). No significant differences were found for food intake, body weight gain, culling rate and carcass performance among the three treatments. Examination of the morphology of the intestinal wall did not show significant differences among treatments. Nevertheless, analysis of the caecal microbiota revealed that only supplementation with combined EOs significantly increased bacterial diversity and some representative probiotic bacteria, particularly *Streptococcus* and *Bifidobacterium* (Xue et al., 2020).

The role of oregano EO as a food supplement has been investigated in farmed animals since this EO, with the major active compounds carvacrol and thymol, has been reported to have antimicrobial and antioxidative properties resulting in improved intestinal barrier function and weight growth in pigs and poultry. However, its impact on GM still remains unclear. Analysis of health records showed that farmed piglets supplemented with oregano EO had a significantly reduced need of therapeutic treatment and overall reduced mortality. In sows and piglets, the GM structure and composition varied considerably over time: sows supplemented with oregano EO during lactation showed an increase in the relative abundance of Lactobacillaceae, Fibrobacteriaceae and Akkermansiaceae, and the analysis of the piglet GM, at two and 4 weeks of age, showed a relative decrease in Enterobacteriaceae and an increase in butyrate producers (from Lachnospiraceae family) at both timepoints. The hypothesis to explain these findings was that this GM modulation in piglets was dependent on maternal microbial transfer (Hall et al., 2021).

Table 2summarize the major effects of fungus, plants and EO molecules on the GM modulation in humans and animal models of diseases.

CONCLUSION AND PROSPECTIVE

There is no doubt that GM modulation represents a therapeutic frontier for the prevention and treatment of many different diseases. Probiotics are the most widely used type of supplement to date for this purpose, even if not always supported by scientific clinical data. On the other hand, the correct intake of prebiotics can be easily achieved with a diet that includes a large consumption of vegetables, but their supplementation requires further studies that define which types and which dosages. Postbiotics have so far shown more limitations than real therapeutic successes. The potential role of fungi and medicinal plants in GM modulation has been tested and analyzed only for a few of them and mainly on animal models. EOs or their single components are widely used in animal breeding in an attempt to reduce the use of antibiotics in meat farms. Their eubiotic effect on GM is demonstrated and, at the doses used, no side effects related to their toxicity appear to be evident. They are certainly able to positively modulate the human GM as well, selectively acting on pathobionts, without altering or even improving the fraction of health-associated commensals. However, new human clinical studies on EO or their single compounds are needed to verify the possibility of being specifically used in the context of metabolic pathologies or diseases in which dysbiosis plays a key role in the pathogenesis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Original raw paired-end sequence data are available in NCBI data base with BioProject accession number PRJNA795336.

AUTHOR CONTRIBUTIONS

Conceptualization, ES, ST, and MCV; writing—original draft preparation, ES, VI, RS, ST, PA, DAy, DAz, and MV; writing—review and editing, ES, ST, and MV. All authors have read and agreed to the published version of the manuscript.

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Ferrostatin-1 Ameliorates Liver Dysfunction via Reducing Iron in Thioacetamide-induced Acute Liver Injury in Mice

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Background and Aims: Hepatic iron overload always leads to oxidative stress, which has been found to be involved in the progression of liver disease. However, whether iron disorder is involved in acute liver disease and the further molecular mechanisms remain unclear.

Methods: A mice model of acute liver injury (ALI) was established via intraperitoneal injection of thioacetamide (TAA) (250 mg/kg/day) for 3 consecutive days. Ferrostatin-1 (Fer-1) was administered intraperitoneally (2.5 μM/kg/day) starting 3 days before TAA treatment. Deferoxamine (DFO) was intraperitoneally injected (200 mg/kg/day) with TAA treatment for 3 days. We further observed the effect of Fer-1 on TAA model with high-iron diet feeding. ALI was confirmed using histological examination and liver function activity. Moreover, expressions of iron metabolism and ferroptosis proteins were measured by Western blot analysis.

Results: The study revealed that the iron accumulation and ferroptosis contributed to TAA-induced ALI pathogenesis. TAA induced prominent inflammation and vacuolar degeneration in the liver as well as liver dysfunction. In addition, protein expression of the cystine/glutamate antiporter SLC7A11 (xCT) and glutathione peroxidase 4 (GPX4) was significantly decreased in the liver, while transferrin receptor 1 (TfR1), ferroportin (Fpn) and light chain of ferritin (Ft-L) expression levels were increased after TAA exposure. As the same efficiency as DFO, pre-administration of Fer-1 significantly decreased TAA-induced alterations in the plasma ALT, AST and LDH levels compared with the TAA group. Moreover, both Fer-1 and DFO suppressed TfR1, Fpn and Ft-L protein expression and decreased iron accumulation, but did not affect xCT or GPX4 expression in the liver. Both Fer-1and DFO prevented hepatic ferroptosis by reducing the iron content in the liver. Furthermore, Fer-1 also reduced iron and reversed liver dysfunction under iron overload conditions.

Conclusion: These findings indicate a role of TAA-induced iron accumulation and ferroptosis in the pathogenesis of ALI model. The effect of Fer-1 was consistent with that of DFO, which prevented hepatic ferroptosis by reducing the iron content in the liver.

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Thus, Fer-1 might be a useful reagent to reverse liver dysfunction and decreasing the iron content of the liver may be a potential therapeutic strategy for ALI.

Keywords: thioacetamide, ferroportin, transferrin receptor 1, ferroptosis inhibitor, acute liver injury, deferoxamine, iron

INTRODUCTION

The liver is one of the vital organs in the body and plays a vital role in the detoxification of foreign substances, secretion of bile for digestion, metabolic functions of various nutrients, and regulation of iron metabolism. The liver is not only the major iron storage site but also where the iron regulation hormone hepcidin is synthesized (Nicolas et al., 2001; Sikorska et al., 2016). Systemic and cellular iron homeostasis is sustained through several main iron-related proteins, including iron importer, exporter and storage proteins. Iron is taken up into cells via transferrin receptor 1 (TfR1) and sequestered by ferritin light chain (Ft-L) (Hentze et al., 2010) or exported out of cells via ferroportin (Fpn). Iron homeostasis is controlled through regulation of duodenal iron absorption, macrophage iron release and hepatocyte iron storage, which is mainly carried out by the iron-related proteins above (Hentze et al., 2010; Ganz 2011).

Acute liver injury (ALI) often develops rapidly and may involve drug-induced liver failure or cholestasis. Animals that develop ALI show enhanced generation of hepatic ROS and enhanced lipid peroxidation with the formation of lipid peroxides. ALI may be caused by viruses, drugs or toxins. Among the various toxins, a thiono-sulfur-containing compound, thioacetamide (TAA), has been used extensively in the development of animal models of ALI (Chu et al., 2001; Bruck et al., 2007) because it induces lipid peroxidation, oxidative stress, and inflammation, ultimately causing functional hepatocyte death and liver dysfunction (Muller et al., 1991; Grek and Arasi 2016; Thawley 2017).

Ferroptosis, which is a recently identified novel form of regulated cell death, proceeds differently from apoptosis, necrosis, and autophagic cell death (Dixon et al., 2012). It is mediated by the iron-dependent oxidative degeneration of lipids and leads to mitochondrial shrinkage, with increased mitochondrial membrane density and outer mitochondrial membrane rupture (Xie et al., 2016). It has been reported that excess cellular iron is the main driver of the Fenton reaction and the production of reactive oxygen species (ROS) (He et al., 2020). Moreover, ferroptosis was demonstrated to be induced by phospholipid peroxidation associated with free iron-mediated Fenton reactions (Hadian and Stockwell 2020). Thus, we hypothesized that TAA-induced ALI is mediated by excess iron, causing substantial oxidative stress/lipid peroxidation and further inducing ferroptosis in hepatocytes, which finally results in liver injury and dysfunction. In this study, iron and ferroptosis were identified as the cornerstones for detecting the mechanism of ALI. TAA was administered intraperitoneally to induce the ALI model in mice. We found that iron content and mobilization were significantly enhanced, as well as liver dysfunction, while protein expression of the cystine/glutamate antiporter SLC7A11 (xCT) and glutathione peroxidase 4 (GPX4) in the liver was decreased in the TAA-induced ALI model. However, both deferoxamine (DFO) and ferrostatin-1 (Fer-1) suppressed the liver dysfunction induced by TAA by reducing iron accumulation in the liver but did not affect xCT or GPX4 expression. Our data suggest that decreasing the iron content in the liver may be a potential therapeutic strategy for ALI.

MATERIALS AND METHODS

Animals and Reagents

All animals were provided by the animal experimental centre of Nantong University. Mice were maintained in stainless steel cages with a relative humidity of 55–60% at 21 ± 2 C with 12-h rotation periods of light and dark. All animal handling procedures were performed according to approved guidelines. All chemicals and reagents used in this study were purchased from Sigma (St. Louis, MO, United States) unless otherwise stated. The ferroptosis inhibitor ferrostatin-1 (Fer-1) was obtained from Selleck Chemicals (S7243, TX, United States).

A Mice Model of Acute Liver Injury

Male Institute of Cancer Research mice (ICR, 8 weeks old) were randomly divided into different groups, including control group, TAA group, Fer-1 pre-treatment and TAA group, or TAA with DFO group (n = 6-9 mice per group). According to previous studies (França et al., 2019; Han et al., 2019; Liu et al., 2021), TAA was injected (i.p., 250 mg/kg/day) for 3 consecutive days to induce acute liver injury. Fer-1 was administered (i.p., 2.5 μM/kg/day) for 3 days before TAA treatment (Wang et al., 2017), and DFO (200 mg/kg/day, i. p) was injected with TAA for 3 days (Mansour 2000; Wu et al., 2011). TAA, DFO and Fer-1 were all dissolved in normal saline. The control group was injected with equal volumes of normal saline solution, using the same injection schedule. To compare the effects of normal-iron diet (NID) and high-iron diet (HID) on TAA-induced ALI model, we next fed the iron content of diets according to previous protocol (Wang et al., 2017). Briefly, male ICR mice were fed with either a standard AIN-76A diet (50 mg Fe/kg; Research Diets, Inc., New Brunswick, United States) or high-iron AIN-76A diet (8.3 g Fe/kg; Research Diets, Inc.) for 14 days. TAA was administered (i.p., 250 mg/kg/day) for 3 consecutive days after HID intervention. The mice were finally anaesthetized with 1% pentobarbital sodium (40 mg/kg body weight, i. p.) and received myocardial perfusion using phosphate-buffered saline (PBS), after which the liver tissues were collected for measurements.

Assessment of Liver Functions

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and glutathione (GSH) were measured following the protocol of an ALT kit (Cat# C009-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), AST kit (Cat# C010-1-1, Nanjing Jiancheng Bioengineering Institute), albumin kit (Cat# A028-1-1, Nanjing Jiancheng Bioengineering Institute), and GSH assay kit (Cat# A006-1-1, Nanjing Jiancheng Bioengineering Institute), respectively. The optical densities were measured with a Synergy 2 multi-mode microplate reader (Agilent Technologies, Inc.). Each sample was measured three times, and the average absorbance values were calculated for every sample. The concentrations in the samples were determined using the standard curves.

Hepatic Histopathological Evaluation

After paraformaldehyde fixation, the hepatic tissue was embedded in paraffin and sectioned with at 5-μm thickness. Liver sections were then mounted onto slides and taken for haematoxylin-eosin (H&E) staining. Briefly, slides were deparaffinized in xylene (5 min, twice) and hydrated by passing through decreasing concentration of alcohol baths (100, 90, 80, 70%) and water, followed by haematoxylin staining for 2 min. The sections were then washed in running tap water until sections "blue" for 5 min. The sections were then rinsed with distilled water, rinsed with 0.1% hydrochloric acid in 50% ethanol, rinsed with tap water for 15 min, stained with eosin for 1 min, and rinsed again with tap water. Next, the slides were dehydrated with 95 and 100% ethanol successively followed by xylene (5 min, twice) for clearing. Finally, sections were mounted with neutral balata and covered with a cover-slip.

Enhanced Perls' Staining Using Diaminobenzidine

Paraformaldehyde-fixed paraffin-embedded tissues were sectioned into 20 μm sections and stored at room temperature. Slides were blocked for nonspecific binding using a solution block, and endogenous peroxidase activity was quenched. 3,3′-Diaminobenzidine (DAB)-enhanced Perls' staining was performed to observe iron accumulation in paraffin-embedded liver sections following the manufacturer's instructions. Briefly, sections of liver tissue were washed with PBS and incubated in freshly prepared Perls' solution (1% potassium ferricyanide in 0.1-M hydrochloric acid buffer) for 1 h, followed by a 15 min incubation in DAB. Slides were immersed for 1 hour and then stained with DAB. All slides were counterstained with haematoxylin and visualized under a DM4000B microscope (Leica, Germany) at a final magnification of ×200. The data were collected from three fields of view per mice, and analyzed semi-quantitatively with ImageJ software. As described previously (Moos and Møllgård 1993), the quantitative analysis of Perls' staining was used to reflect iron deposition levels. Integrated Density of different treatment groups was finally normalized to control group. The analysis was done by an investigator masked to experimental group.

Western Blotting

Proteins were collected and homogenized in RIPA lysis buffer (Beyotime, PRC) and sonicated with a sonifier. The BCA (Pierce, Rockford) detection method was employed to detect protein content. A sample containing 30 μg protein was loaded and run in each well of SDS–PAGE gels. The membranes were incubated with primary antibodies (1:1,000) against TfR1 (Cat. 13–6800, Thermo Fisher Scientific, MA, United States), Fpn1 (Cat. NBP1-21502, Novus, Centennial, CO, United States), ferritin-L (Cat. 10727-1-AP, Proteintech, Chicago, United States), xCT (Cat. 26864-1-AP, Proteintech) and GPx4 (Cat. ab125066, Abcam, Cambridge, United Kingdom) at 4°C overnight. Blots were then incubated with goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibody at a 1:10,000 dilution (Li-Cor, Lincoln Co., Ltd., United States) at room temperature for 1 h. GAPDH (MAB374, Merck, State of New Jersey, United States) monoclonal antibody (1:10,000) was used as a loading control. The band densities of the specific blots were scanned via Odyssey CLx infrared imaging system (LI-COR Biosciences) and analyzed with ImageJ software. Results were shown as the optical density ratio normalized to GAPDH.

Tissues Iron Measurements

Iron in the liver was detected using the tissue iron measurement method as follows. Liver tissue (0.1 g) was obtained, and then, 1 ml of tissue digestive liquid (3 M hydrochloric acid and 0.61 M trichloroacetic acid) was added to the liver for digestion at 65 C for 60 h to ensure complete digestion of the liver tissue. After digestion, the volume of the tissue digestion liquid was fixed to 1.5 ml, and the digestion mixture was centrifuged at 10,000 g for 10 min. The supernatant was removed and collected for detection. Iron developing color working solution was freshly prepared for each experiment, and consisted of 100 mg disodium-4,7-diphenyl-1,10-phenanthroline disulfonate (Cat. 146617, sigma, State of New Jersey, United States), 60 ml ddH2O, 1.429 ml 70% thioglycollic acid (S8750, sigma, State of New Jersey, United States), supplement with ddH2O to 100 ml. A 96-well plate was further used for detection, and 200 ml iron developing colour working solution was added to each well. Then, 10 ml ddH2O, 10 ml tissue digestion solution or 10 ml iron standard solution (500 μg/dl) was added to different wells. Finally, 10 ml of sample was added to the sample well, fully mixed, and incubated at room temperature for 10 min. Absorbance was measured at 535 nm. The method of iron quantification was according to the formula, iron content (Ug/g Tissue wet weight) = OD/tissue weight × (1.5–0.25 × tissue weight) × (1/iron standard 500 OD × 4.77).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8.0 software. All the data in this study are shown as the mean ± SEM. Data of two groups were analyzed for statistical significance with Student's t-test (non-directional). Variations between the means in multiple groups were analysed via one-way analysis of variance, and then, Tukey's post-hoc test was performed for multiple comparisons. A probability value of $p < 0.05$ was viewed as statistically significant.

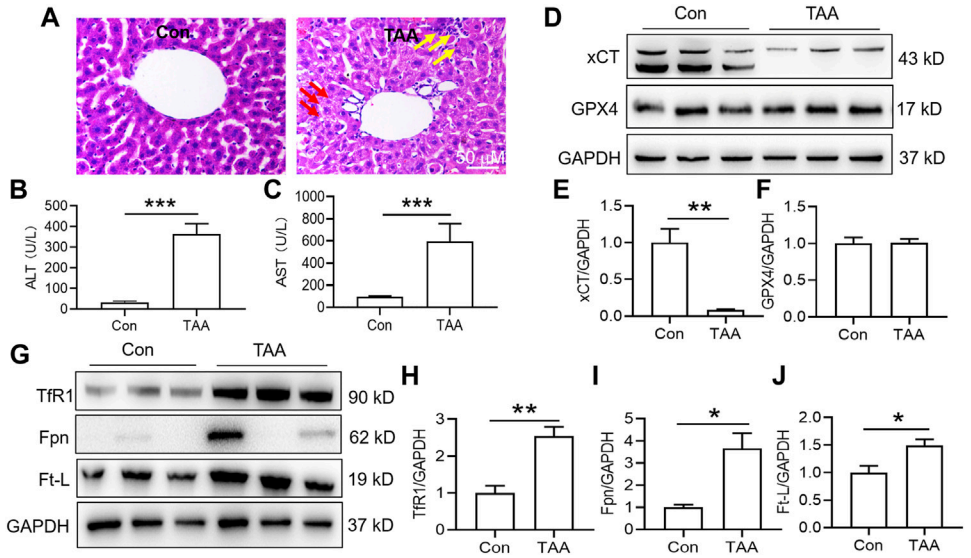

FIGURE 1 | TAA induced liver dysfunction, suppressed anti-ferroptosis-related protein expression and enhanced iron-related protein expression in the liver. **(A)** Liver injury was assessed by H&E staining and histological examination in mice with or without TAA administration for 3 days (bar = 50 μm). TAA induced prominent inflammation (yellow arrow) and vacuolar degeneration (red arrow) in the liver within 3 days compared with the control group. **(B)** Plasma ALT and **(C)** AST levels in control and TAA-injected mice. **(D)** Western blotting analysis of xCT and GPX4 expression in the livers of mice with or without TAA treatment. **(E–F)** Relative protein expression levels of xCT and GPX4. **(G)** Western blotting analysis of TfR1, Fpn and Ft-L expression in the livers of mice with or without TAA treatment. **(H–J)** Relative protein expression levels of TfR1, Fpn and Ft-L. All data are presented as the mean ± SEM (n = 6-9 mice per group); $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the indicated group.

RESULTS

TAA induced liver dysfunction, suppressed anti-ferroptosis-related protein expression and enhanced iron-related protein expression in the liver.

Injection of TAA induced prominent inflammation and vacuolar degeneration in the liver within 3 days compared with the control group (**Figure 1A**). TAA induced obvious liver dysfunction, which was reflected by increased plasma ALT and AST levels in the TAA model (**Figures 1B,C**, $p < 0.001$). In addition, TAA significantly reduced anti-ferroptosis-related protein (xCT) expression but not GPX4 expression compared to the control (**Figures 1D–F**). In addition, TAA induced a significant increase in iron-related proteins, including TfR1, Fpn and Ft-L expression, compared to the control (**Figures 1G–J**, $p < 0.05$ or $p < 0.01$).

Both Fer-1 and DFO suppressed TAA-induced liver dysfunction but did not affect anti-ferroptosis-related protein expression in the liver.

In comparison with the control, injection of TAA induced a profound increase in plasma levels of ALT and AST concomitant with a marked increase in the plasma LDH level. Pre-administration of Fer-1 for 3 days significantly decreased TAA-induced alterations in the plasma ALT, AST and LDH levels compared with the TAA group (**Figures 2A–C**, $p < 0.001$). TAA significantly reduced xCT but not GPX4 expression in the livers of mice. However, pre-administration of Fer-1 did not affect xCT or GPX4 expression in the liver compared to TAA-treated mice (**Figures 2D–F**).

Administration of DFO for 3 days with TAA treatment significantly attenuated the TAA-induced increase in plasma ALT, AST and LDH (**Figures 3A–C**, $p < 0.001$). In addition, administration of DFO did not alter xCT or GPX4 expression induced by TAA in the liver compared to TAA-treated mice (**Figures 3D–F**).

Both Fer-1 and Deferoxamine Reduced Thioacetamide-Induced Iron Accumulation in Liver

TAA induced a significant increase in iron uptake, iron export and iron storage in the liver, which were reflected by increased TfR1, Fpn and Ft-L expression, respectively (**Figures 4A–D**). In addition, iron staining of the liver showed that TAA induced significant iron accumulation (**Figures 4E,F**, $p < 0.001$), especially positive centrolobular deposition of iron and liver nonheme iron content (**Figure 4G**, $p < 0.05$). However, Fer-1 pre-treatment reduced the TAA-induced increase in Fpn and Ft-L but did not affect TfR1 expression. In addition, compared to TAA alone, Fer-1 pre-treatment decreased the TAA-induced iron levels and increased iron accumulation in the liver (**Figure 4**).

TAA similarly upregulated TfR1, Fpn and Ft-L expression in the liver (**Figures 5A–D**, $p < 0.01$ or $p < 0.05$), and also induced significant iron accumulation (**Figures 5E,F**, $p < 0.001$) and increased liver iron content (**Figure 5G**, $p < 0.05$). However, DFO pre-treatment before TAA administration significantly decreased Fpn and Ft-L but did not affect TfR1 expression (**Figures 5A–D**). Additionally, DFO decreased the TAA-induced increase in the iron level and iron accumulation in the liver (**Figures 5E–G**).

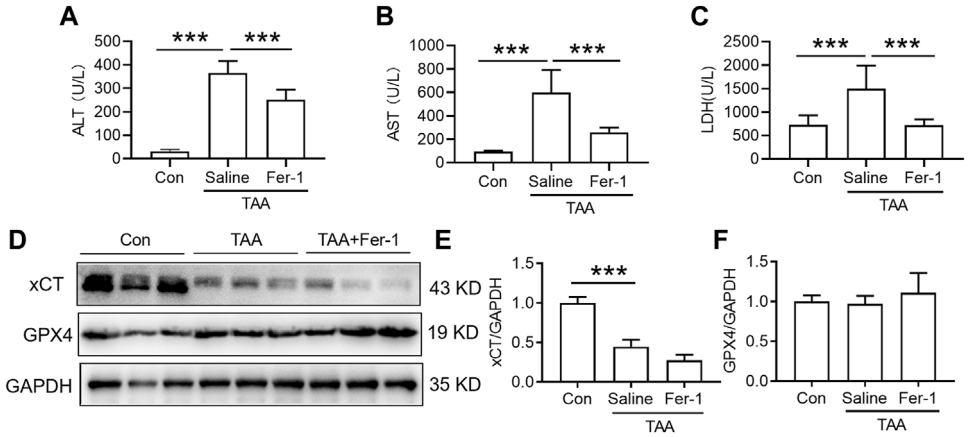

FIGURE 2 | Effects of Fer-1 on liver function and anti-ferroptosis-related protein expression in TAA-induced ALI mice. **(A)** Plasma ALT, **(B)** AST and **(C)** LDH levels in the control and TAA model mice with or without Fer-1 pre-treatment. **(D)** Western blotting analysis of xCT and GPX4 expression in the liver of control and ALI model mice with or without Fer-1 pre-treatment. **(E–F)** Relative protein expression levels of xCT and GPX4 expression in **(D)**. All data are presented as the mean ± SEM (n = 6-9 mice per group); ***$p < 0.001$ versus the indicated group.

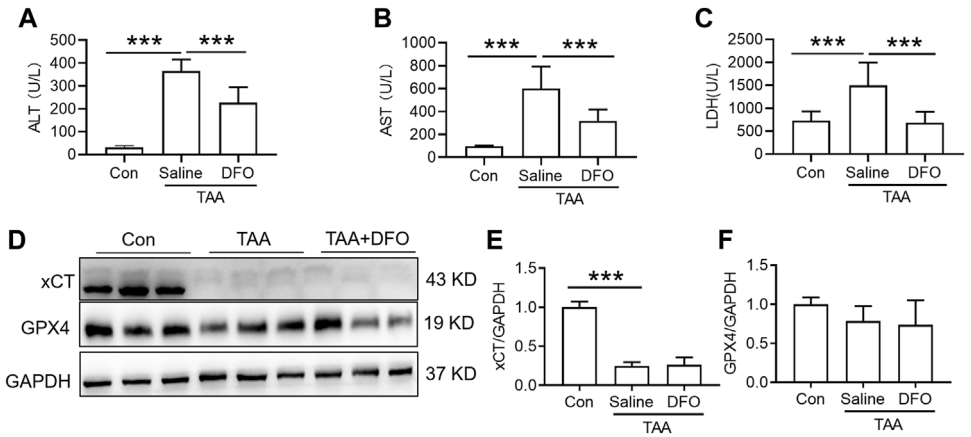

FIGURE 3 | Effects of DFO on liver function and anti-ferroptosis-related protein expression in TAA-induced ALI mice. **(A)** Plasma ALT, **(B)** AST and **(C)** LDH levels in the control and TAA model mice with or without DFO treatment. **(D)** Western blotting analysis of xCT and GPX4 expression in the livers of the control and ALI model mice with or without DFO treatment. **(E–F)** Relative protein expression of xCT and GPX4 in **(D)**. All data are presented as the mean ± SEM (n = 6-9 mice per group); ***$p <$ 0.001 versus the indicated group.

Fer-1 reversed TAA-induced liver dysfunction by reducing iron but not activating anti-ferroptosis-related protein expression in the liver under iron overload conditions.

Compared with NID, HID did not increase the levels of hepatic damage biomarkers (ALT, AST and LDH), and this diet was not inducing the same acute liver damage as TAA (**Figures 6A–C**). In addition, HID downregulated the protein expression of TfR1 (**Figures 6D,E**, $p < 0.05$), but did not influence the expression of Fpn compared to NID (**Figures 6D,F**). Unlike TfR1 level, Ft-L expression was significantly enhanced by HID in the liver (**Figure 6G**, $p < 0.01$). HID-fed mice had developed a significant iron increase than NID mice (**Figures 6H–J**, $p < 0.01$).

Consistent with the effects of TAA under NID conditions, TAA induced profound liver dysfunction, which was shown by an increase in plasma ALT, AST and LDH levels under iron overload conditions (**Figures 6A–C**). Pre-administration of Fer-1 for 3 days before TAA injection significantly attenuated the TAA-induced increase in plasma ALT, AST and LDH compared with the TAA treatment group under high iron conditions (**Figures 6A–C**). Similar to TAA treatment under NID conditions, TAA induced a significant enhancement in TfR1, Fpn and Ft-L expression in the liver (**Figures 6D–G**). Moreover, compared to the HID group, iron staining results suggested that TAA induced significant iron accumulation under high iron conditions (**Figures 6H,I**) and increased liver iron detection (**Figure 6J**). However, pre-treatment of Fer-1 reversed the increases in the expression levels of the above three proteins and iron accumulation in the liver under HID conditions (**Figures 6D–J**).

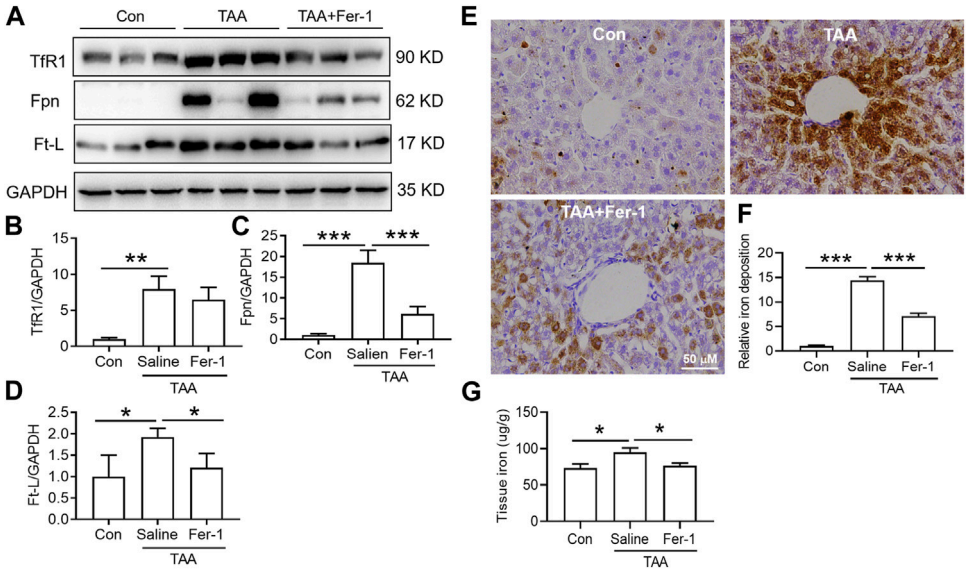

FIGURE 4 | Effects of Fer-1 on iron-related protein expression and iron content in TAA-induced ALI mice. **(A)** Western blotting analysis of TfR1, Fpn and Ft-L expression in the livers of mice after different treatments. **(B–D)** Relative protein expression of TfR1, Fpn and Ft-L in **(A) (E)** Fer-1 suppressed the increase in positive centrolobular iron deposition induced by TAA. **(F)** Semiquantitative iron levels in the mouse livers described in **(E) (G)** Iron content detection in the livers of mice with different treatments. All data are presented as the mean ± SEM (n = 6-9 mice per group); *p < 0.05, **p < 0.01, and ***p < 0.001 versus the indicated group.

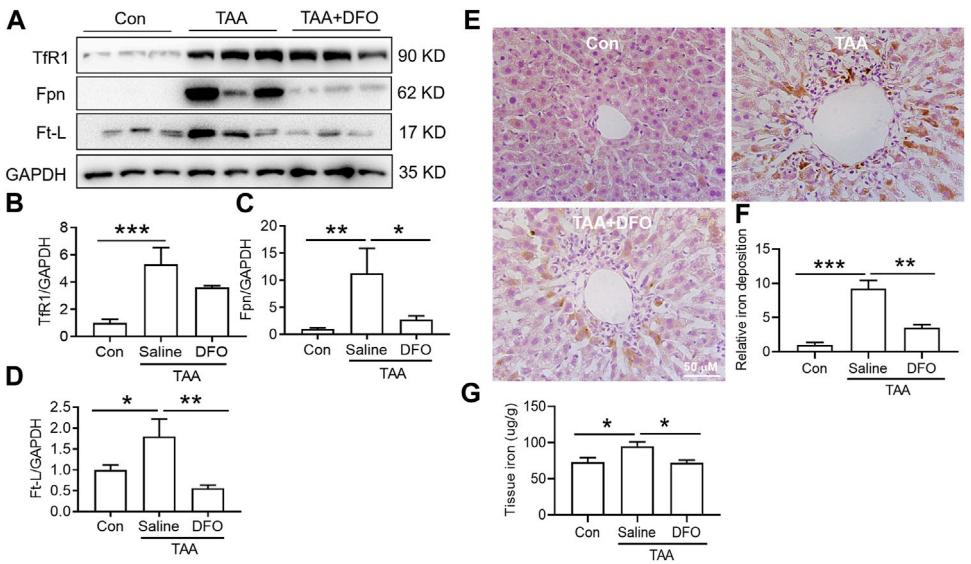

FIGURE 5 | Effects of DFO on iron-related protein expression and iron content in TAA-induced ALI mice. **(A)** Western blotting analysis of TfR1, Fpn and Ft-L expression in the livers of mice with different treatments. **(B–D)** Relative protein exprssion of TfR1, Fpn and Ft-L in **(A) (E)** DFO suppressed the increase in positive centrolobular iron deposition induced by TAA. **(F)** Semiquantitative iron levels of the mice liver in **(E) (G)** Iron content detection in the livers of mice with different treatments. All data are presented as the mean ± SEM (n = 6-9 mice per group); *p < 0.05, **p < 0.01, and ***p < 0.001 versus the indicated group.

DISCUSSION

TAA is a potent toxicant that causes oxidative stress and further induces liver damage, which is highly similar to human acute liver damage (Zargar et al., 2019). The TAA-induced liver injury model has been widely studied for its biochemical and histological effects in animals and is usually used to establish liver injury models (Rahman and Hodgson 2003). However, administration of TAA to mice causes either chronic/acute liver failure or cirrhosis depending on the dose and duration of TAA exposure (Shapiro et al., 2006). In previous studies, high-dosage TAA is known to induce lipid peroxidation and oxidative stress, and the pathological effects are

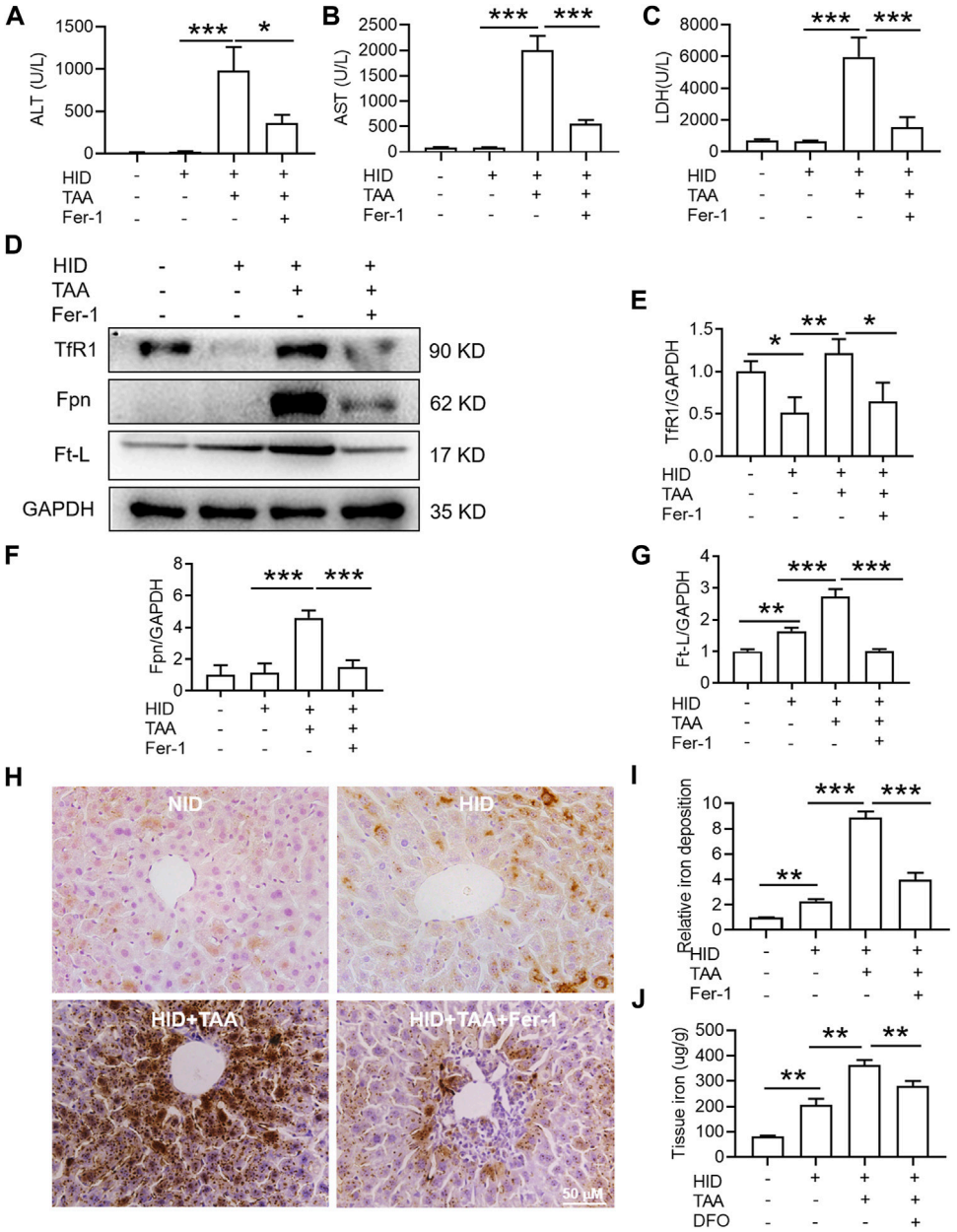

FIGURE 6 | Effects of Fer-1 on liver function, hepatic iron content and anti-ferroptosis-related protein expression in the liver under iron overload conditions. **(A)** Plasma ALT **(B)** AST and **(C)** LDH levels in the control and TAA model mice with or without Fer-1 pre-treatment under high iron diet (HID) conditions. **(D)** Western blotting analysis of TfR1, Fpn and Ft-L expression in the livers of mice with different treatments under HID conditions. **(E–G)** Relative protein expression of TfR1, Fpn and Ft-L in **(D) (H)** Fer-1 inhibited the increase in positive centrolobular iron deposition induced by TAA under HID conditions in the liver. **(I)** Semiquantitative analysis of iron deposition levels in **(H) (J)** Iron content detection in the livers of mice with different treatments. All data are presented as the mean ± SEM (n = 6-9 mice per group); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the indicated group.

mainly limited to ALI rather than causing direct damage to other organs (Muller et al., 1991; Mladenovic et al., 2012). Thus, according to previous studies (França et al., 2019; Han et al., 2019; Liu et al., 2021), we administered 250 mg/kg/day TAA to mice via intraperitoneal injection for 3 days to establish the ALI model in our present study.

Although TAA-induced ALI was primarily provoked by oxidative stress (Zhan et al., 2019), whether it is partially an iron-dependent process is still uncertain. To investigate whether iron disorder is involved in liver dysfunction, we evaluated liver function and liver iron content in TAA-induced ALI mice. Because the liver is the major extraerythrocyte storage organ for iron and most of the iron within the liver is stored in Ft-L, which is often used to indicate iron levels (Ishizaka et al., 2005; Galaris and Pantopoulos 2008). TAA not only significantly induced iron mobilization, which was reflected as increased

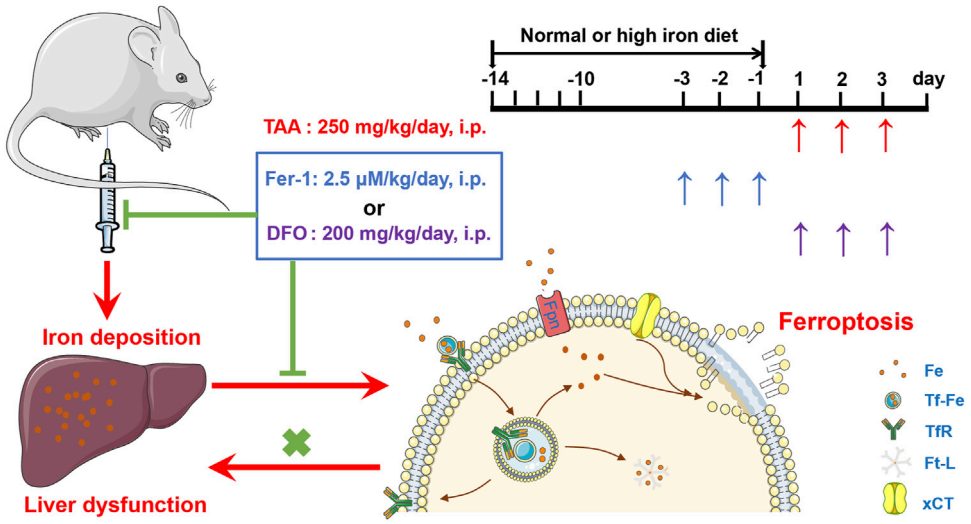

FIGURE 7 | Proposed mechanisms of the ferroptosis inhibitor Fer-1 against the TAA-induced ALI mice model.

transferrin receptor 1 (TfR1), but also increased iron storage, which was reflected as increased ferritin-L (Ft-L) expression in the liver. These results were consistent with those of other studies suggesting that TAA induces iron accumulation in the liver of ALI mice (Ackerman et al., 2015). However, we also noticed that TAA induced the increase of ferroportin (Fpn) expression in the liver, which usually facilitate iron efflux to lower tissue iron. Actually, iron is tightly controlled at cell and systemic levels to prevent both deficiency and overload under physiological conditions. Iron regulatory proteins post-transcriptionally control genes encoding proteins that modulate iron transport, use and storage and are themselves regulated by iron (Gao et al., 2019). Thus, the elevated Fpn may represent an endogenous effort to maintain iron homoeostasis (Anderson and Frazer 2017). It is also understandable that both Fer-1 and DFO decreased iron content, while reducing Fpn in the liver after TAA exposure. Intracellular iron can be stored in the protein shell of ferritin (Ft) as a crystalline core of ferric (Fe^{3+}) ions, and the iron must first be released from the core to catalyze oxidative reactions (Skaper 2019). Thus, Ft is able to restrict the availability of iron to participate in redox active iron species in the cytosol. However, more ferritin subunits are synthesized using translational and transcriptional mechanisms in response to increased cell iron (Badu-Boateng and Naftalin 2019). Increased intracellular ferritin may occur in response to oxidative stress provoked by accumulated iron, which could be result of various stressors such as UV light, increased temperature, and inflammation (Vile and Tyrrell 1993; Tisma et al., 2009). Consistent with previous findings, we found that TAA significantly induced iron increase in the liver and also promoted Ft-L expression.

Although ferroptosis was reported to be involved in the pathogenesis of many injury models (Skouta et al., 2014; Liu et al., 2020), it is still unclear whether ferroptosis is involved in the TAA-induced ALI model. In addition, iron accumulation is considered to be the main cause of ferroptosis initiation (Li et al., 2020). This prompted us to wonder whether TAA induces iron accumulation and ultimately initiates ferroptosis in the liver. The synthetic antioxidant Fer-1 was reported to act as a ferroptosis inhibitor via a reductive mechanism to prevent damage to membrane lipids, thereby inhibiting ferroptosis (Dixon et al., 2012). The iron chelator DFO is widely used to reduce iron in tissue after injection or oral administration (Buss et al., 2003). In the present study, we introduced Fer-1 and DFO to investigate whether ferroptosis occurred because these agents can decrease iron content to achieve an anti-ferroptotic effect in the TAA-induced ALI model. We found that both Fer-1 and DFO rescued liver dysfunction and inhibited iron content and accumulation in the liver. However, it was previously reported that Fer-1 can attenuate oxidative, iron-dependent cancer cell death by blocking cystine import and glutathione production (Skouta et al., 2014). Our data demonstrated that Fer-1 and DFO did not play anti-ferroptotic roles by affecting cystine import or glutathione production in a TAA-induced ALI model. This result may suggest that Fer-1 plays an anti-ferroptotic role similar to DFO in the liver in the ALI model and that the mechanism involves chelation of hepatic iron. Moreover, a recent study demonstrated that Fer-1, in the presence of ferrous iron, produces the most relevant anti-ferroptotic effect by forming a complex with iron (Miotto et al., 2020).

Ferroptosis is a multi-step regulated cell death that is characterized by excessive iron accumulation and lipid peroxidation (Yu et al., 2020). Lipid peroxide accumulation is mainly through the xCT and GPX4-dependent mechanisms (Kim et al., 2021). The process of ferroptosis is also dependent on intracellular iron because the accumulation of iron acts as a catalyst for converting peroxides into free radicals (Kim et al., 2021). Oxidative degradation of lipids occurs when there is depletion of the antioxidant glutathione and a loss of activity of the lipid repair enzyme GPX4. Lipid peroxidation then leads to

cell membrane denaturation (Cao and Dixon 2016). The results were also consistent with other studies, which confirmed that ferroptosis can be induced by a loss of activity of xCT and suppression of GPX4, followed by accumulation of lipid reactive oxygen species (ROS) (Ma et al., 2008; Yang and Stockwell 2016; Latunde-Dada 2017). However, both anti-ferroptotic proteins xCT and GPX4 were not modulated by Fer-1 investigated. It was concluded that the outcome of TAA-induced acute liver injury may depend on the level of hepatic iron concentration and iron overload may exacerbate the injury. However, it's not entirely clear that whether Fer-1 or DFO block iron mobilisation into the liver instead. Thus, more study is necessary to investigate their effects on the expression of molecules that play key roles in iron transport, use and storage.

In addition, the study revealed HID-fed mice had developed a significant iron increase. In addition, HID did not increase the protein expression of TfR1 and Fpn in the same magnitude of TAA. Indeed, HID even downregulated TfR1 compared to NID. Similar with previous research (Weiss et al., 2018), the decreased TfR1 after HID may also represent endogenous feedback to maintain the iron balance. Unlike TfR1 level, Ft-L expression was significantly enhanced by HDI in the liver, which directly indicated increased iron levels. We further found that HID did not increase the levels of hepatic damage biomarkers (ALT, AST and LDH), and this diet was not inducing the same acute liver damage as TAA. However, previous study has revealed that an increase in hepatic iron concentration might exacerbate TAA-induced live injury (Ackerman et al., 2015). We confirmed that TAA aggravated the damage to the liver with iron overload. We further revealed that Fer-1 still had an anti-ferroptotic effect on TAA-induced liver ferroptosis by reducing the iron content in high iron-fed ALI mice. All these results provide new evidence of the anti-ferroptotic effect of Fer-1 in the TAA-induced ALI model, the mechanism of which is mediated by the ability of Fer-1 to reduce iron content and iron accumulation in the liver (**Figure 7**).

Taken together, these findings indicate a role of TAA-induced iron deposition and ferroptosis in the pathogenesis of ALI model. Ferroptosis inhibitor Fer-1 plays a role in rescuing liver injury and dysfunction by reducing liver iron levels and inhibiting TAA-induced ferroptosis in the liver. Similar to DFO, Fer-1 might be a useful reagent to reverse liver dysfunction under iron overload conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Research Ethics Committee of the Institute of Nantong University.

AUTHOR CONTRIBUTIONS

QL and GW conceived, organized, and supervised the work; HJ and WY, performed the experiments; XZ and ML contributed to the analysis of data; QL and GW prepared, wrote and revised the manuscript.

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GLOSSARY

ALI acute liver injury
ALT alanine aminotransferase
AST aspartate aminotransferase
DFO deferoxamine
Fer-1 ferrostatin-1
Fpn ferroportin
Ft-L ferritin-L
GPX4 glutathione peroxidase 4
GSH aseglutathione
TAA thioacetamide
TfR1 transferrin receptor 1
xCT cystine/glutamate antiporter SLC7A11

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Extra Virgin Olive Oil-Based Green Formulations With Promising Antimicrobial Activity Against Drug-Resistant Isolates

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Extra virgin olive oil (EVOO) from *Olea europaea* L. drupes, a cornerstone in the Mediterranean diet, is well known for its nutritional and health properties, especially for prevention of cardiovascular diseases and metabolic disorders. Traditionally, beneficial health effects have been largely attributed to the high concentration of monounsaturated fatty acids, and in recent years, these have also been related to other components including oleacein and oleocanthal. Here, we evaluated, for the first time, the antimicrobial activity of different green extra virgin olive oil-based formulations in natural deep eutectic solvents (NaDESs) emerging as powerful and biocompatible solvents. Specifically, the antimicrobial activity of the EVOO extract, as well as purified oleocanthal and oleacein in two NaDESs (choline/glycerol and choline/propylene glycol), against several drug-resistant clinical isolates and standard microbial strains has been evaluated. The main result was the inhibitory activity of the EVOO extract in choline/glycerol as well as oleacein in choline/propylene glycol toward drug-resistant Gram-positive and -negative strains. Specifically, the EVOO extract in choline/glycerol showed the highest antibacterial activity against several clinical strains of *Staphylococcus aureus*, whereas oleacein in choline/propylene glycol was the most effective toward various clinical strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. In addition, all the formulations tested were effective against *Candida* spp. In conclusion, our results suggest EVOO-based formulations in NaDESs as an interesting strategy that may help in reducing the risk of development of drug resistance. Under this perspective, the usage of NaDESs for the preparation of new antimicrobial formulations may represent a promising approach.

Keywords: antimicrobial activity, extra virgin olive oil extract, oleocanthal, oleacein, NADES, drug-resistant isolates

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INTRODUCTION

Antibiotic resistance is currently one of the greatest threats to human health resulting in an increased number of deaths caused by once curable infections (Huemer et al., 2020). Globally, antibiotic-resistant pathogens are responsible for approximately 700,000 deaths/year, and 10 million deaths/year are expected by 2050, a number greatly exceeding deaths from cancer (O'Neill, 2014; de Kraker et al., 2016; Aslam et al., 2018). However, the estimated number of deaths may more likely increase

following the ongoing coronavirus disease 2019 (COVID-19) pandemic, characterized by elevated antibiotic use in patients infected with SARS-CoV-2 and by the exponential growth in disinfectant use (Ansari et al., 2021).

Over the years, the widespread use of antibiotics has led to an increased incidence of bacterial resistance, beginning with the emergence of methicillin-resistant *Staphylococcus aureus*, which has rapidly become the most frequently occurring resistant pathogen identified in many parts of the world, including Europe (Guo et al., 2020). Following, one recent issue is the increasing prevalence of extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae all over the world, further limiting treatment options (Wilson and Török, 2018). For example, ESBL-producing *E. coli* are resistant to penicillins and most cephalosporins and are often co-resistant to other antimicrobial classes, such as trimethoprim–sulfamethoxazole, quinolones, and aminoglycosides (De Oliviera et al., 2020; Kakoullis et al., 2021). Among the pathogens with growing multidrug resistance, the WHO included ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) against which new antibiotics are urgently needed (World Health Organization, 2017; De Oliveira et al., 2020; Ma et al., 2020).

In this scenario, currently available antibiotic treatments often have limited or no efficacy, and novel therapeutic approaches need to be investigated. Efforts are now focusing on natural products, considered extremely interesting and promising for the preparation of pharmacological formulations and nutraceuticals (Sessa et al., 2015; Sharifi-Rad et al., 2020; Okagu et al., 2021; Oliveira et al., 2021). Recently, the interest in nutraceuticals is growing as most of them possess multiple therapeutic properties, including antimicrobial, antioxidant, anti-inflammatory, and anticancer activities (Di Pietro et al., 2013; Sessa et al., 2017a; Sessa et al., 2017b; Filardo et al., 2020; Mattioli et al., 2020; Ayatollahi et al., 2021). In this regard, extra virgin olive oil (EVOO) obtained from cold pressure of *Olea europaea* L. drupes, a cornerstone in the Mediterranean diet, is well known for its nutritional properties and health effects, especially for the prevention of cardiovascular diseases and metabolic disorders. In fact, consumption of olive oil is able to reduce lipid and DNA oxidation; ameliorate lipid profile and insulin resistance, endothelial dysfunction, and inflammation; and lower blood pressure in hypertensive patients (De Santis et al., 2019; Romani et al., 2019; Serreli and Deiana, 2020).

Traditionally, health properties have been largely attributed to the high concentration of monounsaturated fatty acids (98–99% of the total weight of EVOO); however, in recent years, beneficial effects have also been related to other components, particularly polyphenols with promising antimicrobial properties. Specifically, hydroxytyrosol, tyrosol, oleuropein, and several EVOO extracts have been shown to have antibacterial activity against several oral and foodborne pathogens, as well as against some pathogens responsible for hospital and community infections (Medina et al., 2006; Karaosmanoglu et al., 2010; Nazzaro et al., 2019). Other EVOO compounds, such as the secoiridoids oleocanthal and oleacein, belonging to polyphenols, are nowadays two of the most interesting bioactive natural products under investigation. Both oleocanthal and oleacein have been described to exert anti-inflammatory effects, selective cytotoxicity for cancer cells, and promising neuroprotective activity (Pang and Chin 2018; Lonzano-Castellón et al., 2020; Emma et al., 2021; Gabbia et al., 2021). Interestingly, their antibacterial effects toward several oral pathogens and a *Candida albicans* strain have recently been described (Karygianni et al., 2019).

Despite promising pharmacological activities, oleocanthal and oleacein cannot be easily obtained *via* chemical synthesis, and their isolation and purification from EVOO are expensive with low yields. Recently, a green route for obtaining high yields of oleocanthal and oleacein from EVOO, *via* natural solvents, namely, natural deep eutectic solvents (NaDESs), emerging as powerful and biocompatible solvents, has been proposed (Francioso et al., 2020). Indeed, this is an excellent means for complete dissolution and extraction of a wide range of non-polar and polar compounds.

Here, we evaluated, for the first time, the antimicrobial activity of different green extra virgin olive oil-based formulations in NaDESs. Specifically, the antimicrobial activity of the EVOO extract, as well as purified oleocanthal and oleacein in NaDESs against several drug-resistant clinical isolates and standard microbial strains, has been evaluated.

MATERIALS AND METHODS

Antimicrobial Agents

Extra virgin olive oil (EVOO) (*Olea europaea* L., Coratina cultivar) was obtained from a local market in the Puglia region (Italy). The polyphenolic fraction was extracted as described in Francioso et al. (2020). Briefly, NaDESs were prepared by mixing two components (choline:glycerol 1:1.5 molar ratio and choline:propylene glycol 1:3.3 molar ratio) at 70°C under magnetic stirring for 1 h. After cooling, EVOO was added to the NaDESs in the ratio of 1:20 v/v (NaDES:EVOO). The extraction was carried out under magnetic stirring at room temperature for 15 min and then transferred *via* a separatory funnel for decantation and phases separation. The extract was analyzed to determine the total polyphenol content and polyphenolic composition.

In addition, two pure compounds purified from EVOO (following the method described in Francioso et al., 2020, provided by Active-Italia srl), namely, oleocanthal and oleacein, were dissolved in NaDES (choline:glycerol, 1:1.5 molar ratio; choline:propylene glycol, 1:1.5 molar ratio) or in methanol and tested for their antimicrobial activity.

Determination of Total Phenols

Total phenols were determined by the Folin–Ciocalteu assay as described by Singleton and Rossi (1965). Briefly, the reaction solution was prepared by mixing 790 µl of distilled water with 10 µl of standard, sample, or blank. To these, 50 µl of Folin–Ciocalteu reagent (Merck KGaA, Darmstadt, Germania) was added, incubated for 3 min at room temperature, and then

150 µl of 20% (w/v) Na_2CO_3 was added. After 2 h of incubation, the absorbance at 760 nm was measured using a Hitachi U2000 spectrophotometer (Hitachi, Tokyo, Japan). The results were expressed as gallic acid equivalents (GAE).

Polyphenolic Analysis by UPLC-DAD/MS

Polyphenolic analysis of the extracts was performed on a Waters Acquity H-Class UPLC system, as previously described by Francioso et al. (2020). The chromatographic system was coupled to a photodiode array and a single-quadruple mass detector with an electrospray ionization source. Chromatography was performed on a reverse-phase C18 column (Phenomenex Kinetex, 100 mm × 2.1 mm i.d., 2.6 µm particle size). Solvent A was 0.1% aqueous formic acid (Merck), and solvent B was 0.1% formic acid in Methanol (UPLC gradient grade, Merck). The flow rate was 0.5 ml/min, and the column temperature was set at 35°C. Elution was performed with a linear gradient from 2 to 100% B in a total time of 17 min including re-equilibration. The samples were diluted in the mobile phase and injected through the needle. The photodiode array detector was set up in the range of 200–600 nm. Mass spectrometric detection was performed in the negative electrospray ionization mode, using nitrogen as the nebulizer gas. Analyses were performed in the Total Ion Current (TIC) mode with a mass range of 50–1000 m/z. The capillary voltage was 0.8 kV, cone voltage 15 V, ion source temperature 120°C, and probe temperature 600°C. Compounds were identified by retention time, m/z, UV-Vis spectrum, and comparing them with commercially available standards (obtained from Merck). Quantification of each compound was performed by using standard calibration curves in the range of 0.1–2 nmol.

Microbial Strains and Cultures

The antimicrobial activity was investigated against a representative range of standard bacterial strains and drug-resistant bacterial clinical isolates. Specifically, standard strains included Gram-positive and Gram-negative bacteria [*Staphylococcus aureus* (*S. aureus*) ATCC 6538, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 15442, *Escherichia coli* (*E. coli*) ATCC 10536, *Salmonella choleraesuis* (*S. choleraesuis*) ATCC 10708]. Resistant bacterial strains included clinical isolates of *S. aureus*, *P. aeruginosa*, and *β*-lactamase (ESBL)-producing strains such as *E. coli* and *Klebsiella pneumoniae* (*K. pneumoniae*). Last, yeasts such as *Candida albicans* (*C. albicans*) ATCC 10231 and a clinical strain of *Candida parapsilosis* (*C. parapsilosis*) were examined. Clinical strains were isolated, at the Microbiology Unit of the University Hospital in Rome, from samples processed during routine analysis and cultured in accordance with guidelines approved by the management of the hospital for routine care purposes. Then, all isolates were identified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF, Bruker, Bremen, Germany). Antimicrobial susceptibility testing was performed by using a VITEK 2 System (bioMérieux, Inc. France) and by a MicroScan WalkAway System 96 Plus (Beckman Coulter S.r.l.), by using antimicrobial panels provided by the manufacturer for Gram-negative (Vitek 2 AST-N397; Microscan NMDRM1) and Gram-positive (Vitek 2 AST-P592; Microscan PM-STA36) bacteria. Results were interpreted according to the EUCAST clinical breakpoints (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_12.0_Breakpoint_Tables.pdf). *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were used as quality control strains.

The source and resistance profile of the microbial strains are described in **Table 1**. According to the European Centre for Disease Prevention and Control (ECDC), multi-drug resistance is defined as a resistance to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012).

Bacterial strains and fungal strains were streaked from stock cultures stored at −80°C onto tryptic soy agar (TSA) and sabouraud dextrose agar (SDA), and incubated, for 24 h, at 37°C and 35°C, respectively.

Antibacterial Susceptibility Testing

The minimum inhibitory concentration (MIC) of the extracts and the pure compounds was determined using microdilution assay in Muller–Hinton (MH) broth in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2019). Briefly, bacterial inocula were prepared by suspending colonies into MHB from 18 to 24 h on TSA plates and standardized to 1×10^8 colony-forming units (CFU)/ml using a photometric device (optical density at 620 nm). Then, bacterial suspensions were further diluted at 1:20 in MHB to yield a bacterial cell density of 5×10^6 CFU/ml.

In 96-well flat-bottom microtiter plates, 100 µL of serial dilutions of the antimicrobial agents were inoculated with 10 µL of inoculum (final bacterial concentration 5×10^5 CFU/ml). Serial two-fold dilutions of EVOO extracts in choline/glycerol and choline/propylene glycol were prepared using MHB with polyphenol concentrations from 7200 µg/ml to 225 µg/ml, and from 4680 µg/ml to 585 µg/ml, respectively.

The concentrations of oleocanthal and oleacein in choline/glycerol were twofold serial dilutions ranging from 5.056 µg/ml to 79 µg/ml and from 3.376 µg/ml to 53 µg/ml, respectively. For oleocanthal and oleacein in choline/propylene glycol, the concentrations ranging from 4528 µg/ml to 71 µg/ml and from 3.232 µg/ml to 50 µg/ml, respectively, were used. Last, for oleocanthal and oleacein in methanol, the concentrations ranged from 9.080 µg/ml to 71 µg/ml and from 6456 µg/ml to 50 µg/ml, respectively.

At the same time, a dilution series of NaDESs (choline/glycerol; choline/propylene glycol) was assayed in order to exclude their potential inhibitory effects.

The MIC value was determined as the lowest concentration of extracts or pure compounds able to inhibit the visible growth of each bacterial strain after 24-h incubation at 37°C (CLSI, 2019). To determine the minimum bactericidal concentration (MBC), 10 µL were collected from each well that contained no bacterial growth and was subcultured on agar plates (Muller–Hinton agar); the lowest concentration capable of inhibiting bacterial growth on agar surface was considered as MBC (Krishnamoorthy et al., 2018; Wijesundara et al. 2021).

In every set of experiments, a positive control (gentamicin or oxacillin 16 µg/ml), negative vehicle control (antimicrobial agent

TABLE 1 | Antibiotic resistance profile of microbial strains.

Strain	Source	Antibiotic resistance
S. aureus 1	Rectal swab	BZP
S. aureus 2	Catheter	BZP, OXA, CLIN, ERT, LEV, RIF
S. aureus 3	Skin swab	BZP, OXA, CLIN, ERT, LEV
S. aureus ATCC 6538	American Type Culture Collection	Sensitive
E. coli 1	Urine	AMC
E. coli 2	Tracheal aspirate	β-Lactamase-producing strain AMC, FEP, CAZ, CIP, P/T, TOB, G; CTX, TMP-SMX
E. coli ATCC 10536	American Type Culture Collection	Sensitive
P. aeruginosa 1	Bronchoalveolar lavage fluid	AK, FEP, CAZ, CIP, IPM, MEM, TOB, P/T, CZA
P. aeruginosa 2	Skin swab	FEP, CAZ, CIP, IPM, P/T
P. aeruginosa ATCC 15442	American Type Culture Collection	Sensitive
K. pneumoniae	Urine	β-Lactamase-producing strain AK, AMC, CTX, FEP, CAZ, CZA, CIP, IPM, MEM, P/T, TOB, FOS, G
S. choleraesuis ATCC 10708	American Type Culture Collection	Sensitive
C. parapsilosis	Tracheobronchol aspirate	Sensitive
C. albicans ATCC 10231	American Type Culture Collection	Sensitive

BZP, benzylpenicillin; OXA, oxacillin; CLIN, clindamycin; ERT, erythromycin; LEV, levofloxacin; RIF, rifampicin; AMC, amoxicillin/clavulanic acid; AK, amikacin; FEP, cefepime; CAZ, ceftazidime; CIP, ciprofloxacin; IPM, imipenem; MEM, meropenem; TOB, tobramycin; P/T, piperacillin–tazobactam; CZA, ceftazidime–avibactam; G, gentamicin, FOS, fosfomycin; CTX, cefotaxime; TMP-SMX, trimethoprim–sulfamethoxazole.

and MHB), and culture control (MHB and bacteria only) were included.

Antifungal Susceptibility Testing

Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) were determined using the broth micro-dilution method according to Clinical Laboratory Standards Institute (CLSI) document M-27A3 (CLSI, 2008). Briefly, fungal inocula were prepared by suspending colonies into NaCl 0.5% from 24 h on SDA plates. The suspended cultures were diluted with RPMI 1640 to a cell density of 1-5 x 10^6 CFU/ml adjusted based on the optical density at 530 nm.

In 96-well flat-bottom microtiter plates, 100 µL of tested antimicrobial dilutions were inoculated with 100 µL of inoculum (final concentration 5×10^2–2.5×10^3 CFU/ml).

The MIC value was determined as the lowest concentration of the extracts or the pure compounds that were able to inhibit the microorganism growth after 24 h of incubation at 35°C. To determine the minimum fungicidal concentration (MFC), 10 µL sample collected from each well without visible growth were subcultured onto agar plates; the lowest concentration capable of inhibiting fungal growth on agar surface was considered as the MFC.

In every set of experiments, a positive control (fluconazole 64 µg/ml), a negative vehicle control (antimicrobial agent and RPMI), and a cultured control (RPMI and bacteria only) were included.

Cytotoxicity Assay

The cytotoxic effect of EVOO extracts in NaDESs was evaluated using primary human keratinocytes (HEka, cat. no. C0055C, ThermoFisher Scientific, United States). Briefly, 4000 cells per well were plated in a 96-well plate, which was then incubated at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium with L-glutamine and glucose supplemented with 10% heat-inactivated fetal bovine serum. Following a 24-h incubation, the cells were exposed to two-fold dilutions of the antimicrobial agents (4680 to 1170 µg/ml), with three wells for each dilution, for 24 h at 37°C. Then, the number of viable cells was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay, as previously described (Mastromarino et al., 2014).

Statistical Analysis

The OD values from the cytotoxicity assay were expressed as mean ± standard deviation (SD) of three replicates from three independent experiments. Two-way ANOVA was performed for the analysis of variance. All statistical calculations were performed in Microsoft Excel software (version 2110), by using the Real Statistics Resource Pack (release 7.9.1, https://www.real-statistics.com, accessed on 15 March 2022). A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Polyphenol Composition of Extra Virgin Olive Oil Extracts in NaDES

EVOO extracts obtained from the Coratina cultivar are among the richest in polyphenols. **Figure 1** reports the chromatographic analyses of polyphenols extracted from EVOO by two different NaDESs, choline/glycerol and choline/propylene glycol. The chromatographic profiles revealed the presence of four main components: the simple phenolic alcohols hydroxytyrosol and tyrosol, and the two secoiridoids oleacein and oleocanthal, along with oleuropein aglycone and other minor products eluted in the last part of the chromatographic gradient.

Quantitative analysis reveals that oleacein and oleocanthal are the predominant components in Coratina EVOO, largely exceeding oleuropein aglycone, tyrosol, and hydroxytyrosol. Interestingly, the NaDESs containing propylene glycol is able to extract oleocanthal more efficiently than the NaDESs

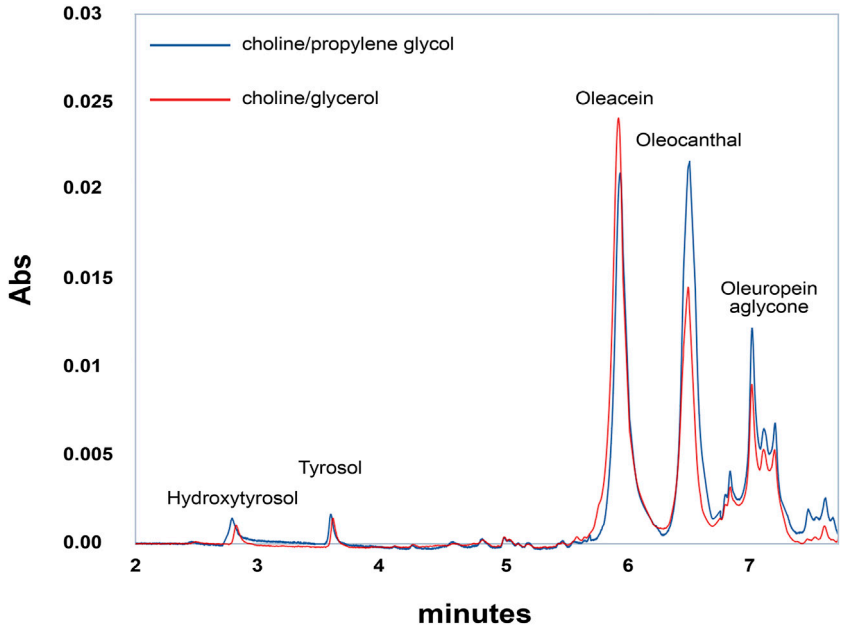

FIGURE 1 | Representative chromatographic profile of a polyphenolic extract from EVOO. Blue line represents choline:glycerol extract. Red line represents choline: propylene glycol extract. EVOO extract was diluted 1:100 in water and 10 µl analyzed *via* UPLC/DAD/MS as described in Materials and Methods. Peak identification was performed on the basis of retention time, UV-Vis, and mass spectra.

TABLE 2 | Polyphenol composition of EVOO extracts in NaDESs.

EVOO extract	Oleocanthal (mM)	Oleacein (mM)	Polyphenols[a] (mM)
Choline/glycerol	5.2	6.6	52.9 (9 mg/ml)
Choline/propylene glycol	9.3	6.3	68.8 (11.7 mg/ml)

[a]*Determined by Folin–Ciocalteu assay and expressed as gallic acid equivalents.*

TABLE 3 | Antibacterial activity of EVOO extracts in NaDESs against Gram-positive standard strains and drug-resistant clinical isolates.

Strain	EVOO extract choline/glycerol (µg/ml)[a]		EVOO extract choline/propylene glycol (µg/ml)[a]	
	MIC	MBC	MIC	MBC
S. aureus ATCC 6538	900	900	1170	2340
S. aureus 1	900	1800	1170	1170
S. aureus 2	900	900	1170	1170
S. aureus 3	900	1800	1170	2340

[a]*Expressed as gallic acid equivalents.*

containing glycerol, due to the less hydrophilic nature of propylene glycol. The polyphenol composition of EVOO extracts is reported in **Table 2**.

Antimicrobial Activity

The two EVOO extracts in NaDESs and two purified bioactive compounds, oleocanthal and oleacein, dissolved in the same NaDESs, were screened. **Tables 3–8** show the MIC and MBC values for each product examined against the bacterial and fungal strains.

Antibacterial Activity of Extra Virgin Olive Oil Extracts

As shown in **Tables 3**, **4**, EVOO extracts, both in choline/glycerol and choline/propylene glycol, had antibacterial activity against Gram-positive and Gram-negative bacteria including standard strains and drug-resistant clinical isolates. Overall, Gram-positive bacteria (MIC 900-1170 µg/ml, MBC 900-2340 µg/ml) were more susceptible to EVOO extracts than Gram-negative bacteria (MIC 1170-2340 µg/ml, MBC 1800-3600 µg/ml). In particular, the antibacterial activity of the EVOO extract in choline/glycerol (MIC 900 µg/ml; MBC 900-1800 µg/ml) toward Gram-positive

TABLE 4 | Antibacterial activity of EVOO extracts in NaDESs against Gram-negative standard strains and drug-resistant clinical isolates.

Strain	EVOO extract choline/glycerol (μg/ml)[a]		EVOO Extract choline/propylene glycol (μg/ml)[a]	
	MIC	MBC	MIC	MBC
E. coli ATCC 10536	1800	1800	1170	2340
E. coli 1	1800	3600	2340	2340
E. coli 2	1800	3600	2340	2340
P. aeruginosa ATCC 15442	1800	3600	1170	2340
P. aeruginosa 1	1800	1800	1170	2340
P aeruginosa 2	1800	1800	2340	2340
S. choleraesuis ATCC 10708	1800	3600	2340	2340
K. pneumoniae	1800	3600	2340	2340

[a]*Expressed as gallic acid equivalents.*

TABLE 5 | Antifungal activity (μg/ml) of EVOO extracts in NaDESs against *Candida* spp.

Strain	EVOO extract choline/glycerol (μg/ml)[a]		EVOO extract choline/propylene Glycol (μg/ml)[a]	
	MIC	MFC	MIC	MFC
C. albicans ATCC 10231	225	450	292	585
C. parapsilosis	225	900	292	585

[a]*Expressed as gallic acid equivalents.*

TABLE 6 | Antibacterial activity of oleocanthal and oleacein in NaDESs against Gram-positive standard strains and drug-resistant clinical isolates.

Strain	Oleocanthal choline/ glycerol (μg/ml)		Oleocanthal choline/ propylene glycol (μg/ml)		Oleacein choline/ glycerol (μg/ml)		Oleacein choline/ propylene glycol (μg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 6538	316	632	283	566	211	422	404	808
S. aureus 1	632	1264	1132	1132	1688	1688	808	1616
S. aureus 2	1264	2528	1132	2264	1688	1688	1616	1616
S. aureus 3	1264	2528	1132	1132	1688	1688	808	1616

TABLE 7 | Antibacterial activity of oleocanthal and oleacein in NaDESs against Gram-negative standard strains and drug-resistant clinical isolates.

Strain	Oleocanthal choline/ glycerol (μg/ml)		Oleocanthal choline/ propylene glycol (μg/ml)		Oleacein choline/ glycerol (μg/ml)		Oleacein choline/ propylene glycol (μg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli ATCC 10536	1264	2528	1132	1132	1688	1688	808	1616
E. coli 1	632	632	566	1132	422	844	404	808
E. coli 2	1264	1264	1132	1132	844	844	202	404
P. aeruginosa ATCC 15442	1264	2528	1132	2264	1688	1688	808	1616
P. aeruginosa 1	632	1264	566	1132	844	844	404	404
P aeruginosa 2	632	632	566	1132	422	422	404	404
S. choleraesuis ATCC 10708	316	632	283	1132	211	422	808	1616
K. pneumoniae	316	632	283	1132	422	422	404	808

bacteria was higher than that of the EVOO extract in choline/propylene glycol (MIC 1170 μg/ml; MBC 1170-2340 μg/ml). For most of the Gram-negative bacteria, the lower MIC value was observed for the EVOO extract in choline/glycerol, whereas the lower MBC value was observed for EVOO extract in choline/propylene glycol.

Interestingly, among the multidrug-resistant strains, the most susceptible bacteria to the EVOO extract in choline/glycerol were

TABLE 8 | Antifungal activity of oleocanthal and oleacein in NaDES against *Candida* spp.

Strain	Oleocanthal choline/ glycerol (μg/ml)		Oleocanthal choline/ propylene glycol (μg/ml)		Oleacein choline/ glycerol (μg/ml)		Oleacein choline/ propylene glycol (μg/ml	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans ATCC 10231	158	632	283	566	211	422	202	404
C. parapsilosis	158	1264	283	1132	211	844	202	808

S. aureus strain 2 (MIC/MBC 900 μg/ml) and *P. aeruginosa* strains 1 and 2 (MIC/MBC 1800 μg/ml). Similarly, the most susceptible bacteria to the EVOO extract in choline/propylene glycol were *S. aureus* strain 2 (MIC/MBC 1170 μg/ml) and *P. aeruginosa* strain 1 (MIC 1170 μg/ml, MBC 2340 μg/ml). More interestingly, ESBL-producing and carbapenem-resistant *K. pneumoniae* as well as ESBL-producing *E. coli* strain 2, resistant to several antibiotic classes (MIC 1800 μg/ml), were also susceptible to the EVOO extract in choline/glycerol, whereas the MBC value against both strains was the lowest for the EVOO extract in choline/propylene glycol (MBC 2340 μg/ml).

Antifungal Activity of EVOO Extracts

EVOO extracts, both in choline/glycerol and choline/propylene glycol, had antifungal activity against *C. albicans* ATCC 10231 and clinical isolate of *C. parapsilosis* with MIC and MFC values of 225–292 μg/ml and 450-900 μg/ml, respectively (**Table 5**). Overall, EVOO extracts in choline/glycerol (MIC 225 μg/ml) and in choline/propylene glycol (MIC 292 μg/ml) showed a similar inhibitory activity toward *C. albicans* and *C. parapsilosis*. On the contrary, the MFC value of the EVOO extract in choline/propylene glycol (MFC 585 μg/ml) was lower than that in the EVOO extract in choline/glycerol (MFC 450-900 μg/ml).

Antibacterial Activity of Oleocanthal and Oleacein

As shown in **Tables 6**, **7**, oleocanthal and oleacein, both in choline/glycerol and choline/propylene glycol, had antibacterial activity against Gram-positive and Gram-negative bacteria including standard strains and drug-resistant clinical isolates. Overall, oleacein in choline/propylene glycol displayed the highest antibacterial activity against Gram-negative bacteria, with MIC and MBC ranging from 202 μg/ml to 808 μg/ml, and from 404 μg/ml to 1616 μg/ml, respectively. Specifically, the highest susceptibility was observed for multidrug-resistant ESBL-producing *E. coli* (strain 2) (MIC 202 μg/ml and MBC 404 μg/ml), as well as *P. aeruginosa* strains 1 and 2 (MIC/MBC 404 μg/ml). Interestingly, oleacein in choline/propylene glycol was also active against Gram-positive bacteria, with a MIC range of 404 μg/ml to 808 μg/ml, apart from *S. aureus* strain 2 (MIC 1616 μg/ml), although its MBC value was lower than that of oleacein in choline/propylene glycol.

Last, higher MIC and MBC values for oleocanthal and oleacein in methanol were observed toward standard strains of *E. coli* and *P. aeruginosa*, than the same compounds dissolved in the two NaDESs (**Supplementary Table S1**).

Antifungal Activity of Oleocanthal and Oleacein

Oleocanthal and oleacein, both in choline/glycerol and choline/propylene glycol, had antifungal activity against *C. albicans* ATCC 10231 and clinical isolate of *C. parapsilosis* (**Table 8**). Overall, oleocanthal (MIC 158-283 μg/ml) and oleacein (MIC 202-211 μg/ml) showed a similar inhibitory activity toward *C. albicans* and *C. parapsilosis*. On the contrary, the MFC value of oleacein (MFC 404-844 μg/ml) was lower than that of oleocanthal (MFC 566 -1264 μg/ml).

More importantly, oleacein in choline/propylene glycol was the most effective toward *C. albicans* and *C. parapsilosis* with MIC values of 202 μg/ml for both strains and MFC values of 404 μg/ml and 808 μg/ml, respectively.

Antimicrobial and Antifungal Activity of NaDESs

To assess the potential inhibitory effects of choline/glycerol and choline/propylene glycol against all microbial strains examined, NaDES concentrations ranging from 25 to 0.77% (v/v) were also investigated. Both choline/glycerol and choline/propylene glycol did not show antimicrobial or antifungal activity at or below a concentration of 25% (v/v).

Cytotoxic Activity

In order to assess the cytotoxic effect of EVOO extracts in NaDESs as well as of oleacein and oleocanthal in NaDES, HEKa cells were incubated with increasing concentrations of each product for 24 h, and then cell viability was measured by the MTT assay. No significant cytotoxicity was observed at the concentrations that showed antimicrobial activities (**Supplementary Figure S1**).

DISCUSSION

To our knowledge, this is the first study evaluating the antimicrobial activity of EVOO extracts using as two natural solvents NaDESs (choline/glycerol and choline/propylene glycol). In addition, its main components, oleacein and oleocanthal in choline/glycerol and choline/propylene glycol, have also been investigated. The main result of clinical importance was the inhibitory activity of the EVOO extract in choline/glycerol and that of oleacein in choline/propylene glycol toward drug-resistant Gram-positive and -negative bacterial strains. Specifically, the EVOO extract in choline/glycerol showed the highest antibacterial activity against several clinical strains of *S. aureus*, whereas oleacein in choline/propylene glycol was the

most effective toward various clinical strains of *E. coli*, *P. aeruginosa* and *K. pneumoniae*.

The highest inhibitory effect of the EVOO extract in choline/glycerol against drug-resistant strains of *S. aureus* may be attributed to the synergistic effect of its main components as detected by UPLC/DAD/MS analysis (oleacein, oleocanthal, and oleuropein aglycone), since, in our study, both oleacein and oleocanthal alone were less active. Also, Karygianni et al. (2019) demonstrated low antibacterial activity of oleacein, oleocanthal, and oleuropein alone toward *S. aureus*, further suggesting that the combination of the components present in our EVOO extract might possess a higher effect.

Of note, the EVOO extract in choline/glycerol was also active toward different drug-resistant Gram-negative strains including *P. aeruginosa*, *E. coli*, and *K. pneumoniae*. Such effects may be due to the ability of choline/glycerol to extract more hydrophilic compounds from EVOO, such as oleacein, that might be more effective against Gram-negative bacteria since their outer membrane surrounding the cell wall has been described to hinder the diffusion of non-polar molecules through the lipopolysaccharide (Vaara, 1992; Denyer and Maillard, 2002).

Even more important is the highest inhibitory effect of oleacein in choline/propylene glycol toward all the multidrug-resistant Gram-negative strains examined, including the ESKAPE pathogens such as *P. aeruginosa* and *K. pneumoniae*. ESKAPE pathogens have been recognized as a considerable public health concern because their resistance has reached alarming levels, requiring urgent intervention as underlined by the most recent WHO reports (World Health Organization, 2017). ESKAPE pathogens are usually encountered in the hospital setting and may become, in the future, a serious threat in community settings where the overuse and misuse of antimicrobial agents are increasing.

The highest antibacterial activity of oleacein in choline/propylene glycol toward multidrug-resistant Gram-negative bacteria may also be due to its NaDES content (2.1–8.4% v/v) since our preliminary experiments showed a lower activity of oleacein in methanol. Such effect is also suggested by evidence in the literature, where oleacein in another solvent (1.1% of DMSO) has been demonstrated to be less active toward Gram-negative strains (Karygianni et al., 2019). A potential mechanism through which choline/propylene glycol may augment the antibacterial effect of oleacein might consist in increased membrane permeability and/or damaged morphology of bacterial cells, facilitating the uptake of the active compound. Indeed, many studies demonstrated that NaDESs significantly increase the bioavailability of bioactive molecules and drugs (Faggian et al., 2016; Sut et al., 2017; Jeliński et al., 2019; da Silva et al., 2021; Mustafa et al., 2021).

In addition to improving the antimicrobial activity, the usage of NaDESs also has additional advantages; NaDESs are characterized by a low environmental impact, combined with the optimization of the extraction of active principles. Indeed, toxic by-products or residual solvents are not generated (Francioso et al., 2020), as also supported by the absence of cytotoxic effects found in our study.

Another interesting finding observed in our study is the marked antifungal activity of all the assayed compounds, including EVOO extract, oleacein as well as oleocanthal in both NaDESs, and *Candida* spp. To date, available antifungal drugs are few, and their widespread use has led to the development of drug resistance in the treatment of *Candida* infections, a problem of growing importance, especially in immunodeficient conditions (Pristov and Ghannoum, 2019).

Overall, our results are another interesting piece of the EVOO puzzle and, mainly, of its antimicrobial properties. Indeed, in the literature, there are mentions of the antimicrobial activity of olive oil extracts on numerous standard microorganisms including *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans*, foodborne pathogens such as, *Listeria* spp., *Salmonella* spp. and several clinical isolates such as *Helicobacter pylori*, *Shigella sonnei*, *Bacteroides* spp., and *Yersinia* spp. (Medina et al., 2006; Romero et al., 2007; Medina et al., 2009; Fei et al., 2018, 2019; Guo et al., 2019; Nazzaro et al., 2019; Guo et al., 2020). Nevertheless, a comparison of the antibacterial activity observed among several studies is quite difficult since different experimental protocols and a diverse variety of olive plants and different types of olive oil (olive oil, virgin olive oil, EVOO), were used. Regarding the chemical composition, it is well known that it depends on different factors such as cultivar, geographic origin, climatic conditions, and processing techniques (De Santis et al., 2019). In addition, the type of bioactive compounds of olive oil extracts is influenced by the chemical properties of the extraction solvent, as evidenced by our study where an EVOO extract rich in oleocanthal and oleacein was obtained by using NaDES.

Oleocanthal and oleacein have already been described to have antimicrobial activity against several standards strains such as *E. coli*, *S. aureus*, *L. monocytogenes*, *H. pylori*, *Pseudomonas fluorescens*, *Enterococcus faecalis*, oral streptococci, and anaerobic pathogenic bacteria as well as against *C. albicans* (Medina et al., 2006; Romero et al., 2007; Medina et al., 2009; Karygianni et al., 2019). In our study, similar MIC values of oleocanthal and oleacein for *E. coli* and lower MIC values for *S. aureus* and *C. albicans* were observed as compared to data of Karygianni et al. (2019), which used the same method for evaluating the antimicrobial activity.

The strength of our study lies in the investigation of several drug-resistant strains, which might better characterize the potential antibacterial activity of EVOO, oleocanthal and oleacein. However, the lack of data regarding the mechanisms by which these natural compounds may inhibit the growth of clinical isolates or cause bacterial cell death represents the main limitations of our study. However, Guo et al. (2019, 2020) found that the antibacterial effect of the olive oil polyphenol extract against several foodborne pathogens such as *L. monocytogenes*, *S. aureus*, and *S. typhimurium* was related to lower intracellular adenosine 5′-triphosphate, cell depolarization, decrease in bacterial protein and DNA, and cell fluid leakage due to destruction of cell morphology.

In conclusion, our study shows that the EVOO extract in choline/glycerol and oleacein in choline/propylene glycol have

potential activity against multidrug-resistant *S. aureus* and several ESKAPE pathogens. In the future, given their broad antimicrobial activity, EVOO-based formulations in NaDES might be an interesting strategy that may help in reducing the risk of development of drug resistance. Under this perspective, the usage of NaDES for the preparation of new antimicrobial formulations may represent a new approach that deserves further research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RS and LM conceived and designed the study. AF and RM performed the extraction of extra virgin olive oil and chemical analysis. MD, SF and GR performed microbiological experiments. RS, MD, SF, AF, RM, and LM analyzed and interpreted the data. GR, RS and LM contributed reagents/materials/analysis tools. All authors contributed to writing and revising the manuscript and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.885735/full#supplementary-material

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REVIEW
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New Drugs for Hepatic Fibrosis

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The morbidity and mortality of hepatic fibrosis caused by various etiologies are high worldwide, and the trend is increasing annually. At present, there is no effective method to cure hepatic fibrosis except liver transplantation, and its serious complications threaten the health of patients and cause serious medical burdens. Additionally, there is no specific drug for the treatment of hepatic fibrosis, and many drugs with anti-hepatic fibrosis effects are in the research and development stage. Recently, remarkable progress has been made in the research and development of anti-hepatic fibrosis drugs targeting different targets. We searched websites such as PubMed, ScienceDirect, and Home-ClinicalTrials.gov and found approximately 120 drugs with anti-fibrosis properties, some of which are in phase II or III clinical trials. Additionally, although these drugs are effective against hepatic fibrosis in animal models, most clinical trials have shown poor results, mainly because animal models do not capture the complexity of human hepatic fibrosis. Besides, the effect of natural products on hepatic fibrosis has not been widely recognized at home and abroad. Furthermore, drugs targeting a single anti-hepatic fibrosis target are prone to adverse reactions. Therefore, currently, the treatment of hepatic fibrosis requires a combination of drugs that target multiple targets. Ten new drugs with potential for development against hepatic fibrosis were selected and highlighted in this mini-review, which provides a reference for clinical drug use.

Keywords: anti-hepatic fibrosis drug, hepatic fibrosis, HSCs, inflammation, oxidative stress

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INTRODUCTION

Hepatic fibrosis is one of the most important manifestations of chronic liver injury (Roehlen et al., 2020). At present, the mechanism of its occurrence has not yet been clarified and there is a lack of effective treatment drugs (Gilgenkrantz et al., 2021). Hepatic fibrosis is a necessary process for most chronic liver diseases to develop into cirrhosis. Hepatic fibrosis is a pathological process of abnormal deposition of extracellular matrix (ECM) in liver tissue caused by a persistent injury-repair response, which further leads to abnormal changes in liver structure and function (Boyer-Diaz et al., 2021). The activation of hepatic stellate cells (HSCs) is the central link in the occurrence of hepatic fibrosis, and the inflammatory response to liver cell injury plays a key role in the development of fibrosis (Chen et al., 2021).

Hepatic fibrosis is caused by a variety of etiological factors, including alcoholism, viral hepatitis, autoimmune hepatitis, non-alcoholic steatohepatitis (NASH), primary biliary cirrhosis, and primary bile duct cirrhosis (intrinsic and extrinsic factors). Hepatic fibrosis is an inflammation disorder, and cytotoxicity, liver damage, and excessive accumulation of fat can cause the liver inflammatory

TABLE 1 | List of drugs currently being evaluated in phase II and phase III clinical trials.

Drug(s)	Mechanism	Research Unit	Research State	Trial Identification
Belapectin	Gal-3	Galectin Therapeutics	II	NCT02421094
Cenicriviroc	CCR2/5	Takeda	III	NCT03028740
Elafibranor	PPAR-α/δ	Genfit	III	NCT02704403
Emricasan	Pan-caspase	Conatus Pharmaceutical	IIb	NCT02686762
Liraglutide	GLP-1	Novo Nordisk	III	NCT02654665
Obeticholic acid	FXR	Intercept	III	NCT02548351
Pentoxifylline	TNF-α	US Pharm Holdings	II	NCT02283710
Pirfenidone	PDE	Marnac	II	NCT02161952
Simtuzumab	LOXL2	Gilead	II	NCT01707472
Sorafenib	VEGFR-2/PDGF-β	Bayer	III	NCT01849588

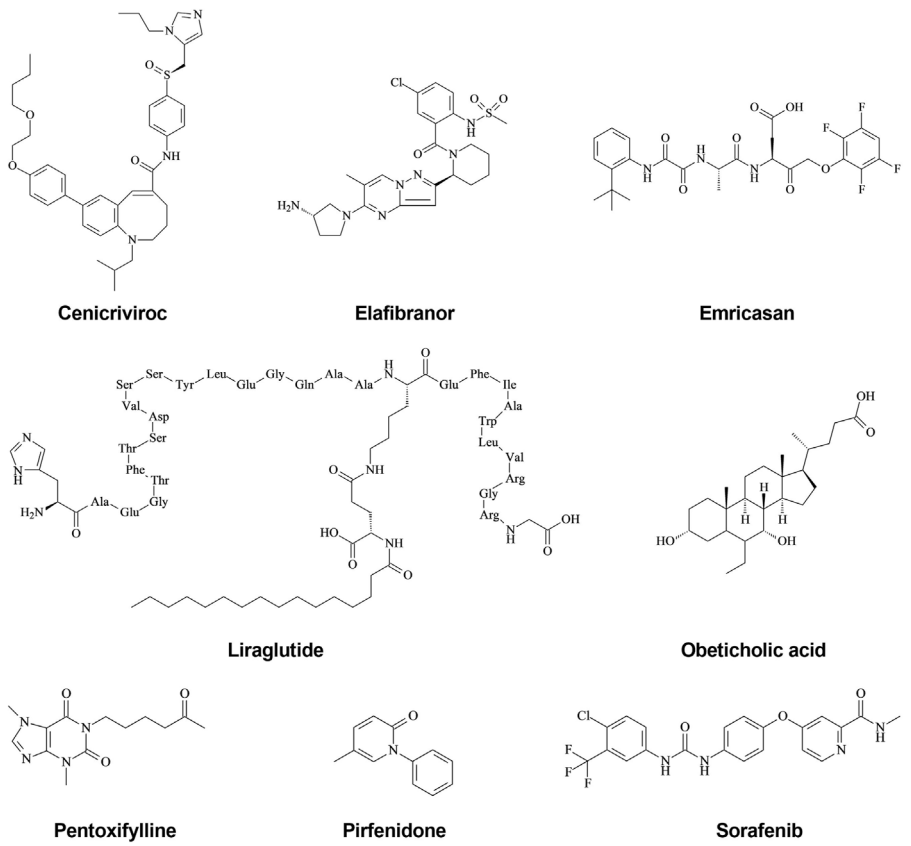

FIGURE 1 | Structural formula of new drugs with anti-hepatic fibrosis effects. (1) Cenicriviroc is a dual antagonist of CCR2/5, which also inhibits both HIV-1 and HIV-2, and displays potent anti-hepatic fibrosis activity. (2) Elafibranor is a double agonist of PPARα/δ, and both animal experiments and clinical trials have found that it has anti-hepatic fibrosis activity. (3) Emricasan is an irreversible pan-caspase inhibitor, which is currently in a phase 2 clinical trial to test its efficacy in treating liver injury and fibrosis, although its clinical trial results are controversial. (4) Liraglutide is a GLP-1 receptor agonist used clinically to treat type 2 diabetes, which functions by ameliorating the progression of NAFLD in patients with T2DM. (5) Obeticholic acid is a potent, selective, and orally active FXR agonist; FXR has been shown to reduce inflammatory mediator expression in HSCs via the induction of PPARγ, suggesting that FXR is an ideal target for the treatment of hepatic fibrosis. (6) Pentoxifylline is an orally active non-selective PDE inhibitor, with anti-inflammatory and anti-proliferation effects. PDE has a strong anti-fibrosis effect but needs more in-depth research. (7) Pirfenidone has broad-spectrum anti-fibrosis effects and is the first drug to demonstrate some efficacy for IPF, which was approved by the FDA in 2008. The mechanism of action of Pirfenidone may be related to the reduction of TGF-β-induced signal transduction pathways. (8) Sorafenib is a multikinase inhibitor of Raf-1, B-Raf, and VEGFR-2, an inhibitor of tyrosine protein kinases that targets the Raf/Mek/Erk pathway. Animal experiments have shown that Sorafenib has anti-fibrosis effects, and the mechanism may be related to inhibiting the TGF-β1/Smad3 pathway.

response. Inflammatory cytokines released by inflammatory cells promote the activation of HSCs, which leads to an increase in ECM release and ultimately causes hepatic fibrosis. Intrinsic factors encompass genetic alterations of cellular pathways leading to the activation of inflammatory pathways such as nuclear factor kappa-light-chain-enhancer of activated B cells

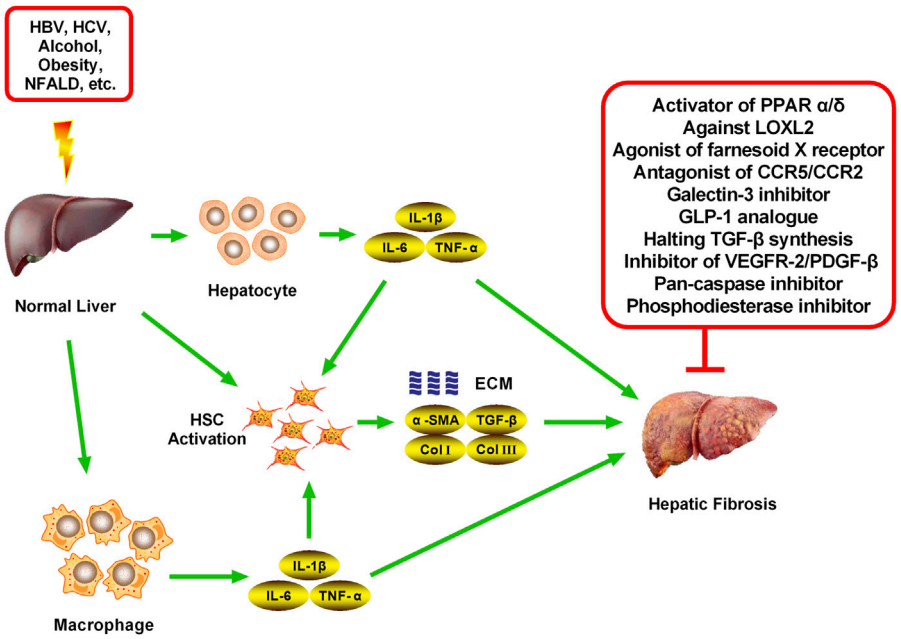

FIGURE 2 | Main targets of new drugs against hepatic fibrosis. Hepatic fibrosis can be induced by various factors, including HBV, HCV, alcohol, obesity, and NFALD, all of which can stimulate the normal liver to induce an inflammatory response. Various cells in the liver, mainly hepatocytes, macrophages, and HSCs, secrete inflammatory cytokines (mainly IL-1β, IL-6, and TNF-α) after receiving stimulation. A large number of inflammatory cytokines continuously stimulate HSCs, inducing activation, proliferation, and the secretion of many fibrosis cytokines, including α-SMA, TGF-β, collagen-I, and Collagen-III, which are important components of the ECM. The chronic accumulation of ECM eventually leads to hepatic fibrosis. Inhibiting the activation and proliferation of HSCs and reducing the accumulation of ECM are the most important methods to reverse hepatic fibrosis. Numerous recent studies have found many targets for inhibiting the process of hepatic fibrosis, which has led to the development of novel therapeutic drugs, mainly activators of PPAR α/δ, agonists of FXR, antagonists of CCR2/5, Galectin-3 inhibitor, GLP-1 analog, inhibitors of VEGFR-2/PDGF-β, LOXL2 inhibitor, Pan-caspase inhibitor, a Phosphodiesterase inhibitor, and TGF-β signaling inhibitors.

(NF-κB), among others. Extrinsic components include inflammatory pathways activated by the liver microenvironment, such as chemokines, cytokines, and adhesion molecules. Although 120 drugs are currently being evaluated for treating hepatic fibrosis, none have yet been approved by the United States Food and Drug Administration (FDA) to treat the disease (Roehlen et al., 2020; Gilgenkrantz et al., 2021). However, many phases II and III clinical trials are ongoing, and a new chapter for treating hepatic fibrosis is expected in the near future (**Table 1**) (**Figure 1**). The main reason for the failure of the 120 drugs is that most are in the stage of animal experiments or clinical trials, with different shortcomings and a lack of adequate data on evidence-based medicine.

BELAPECTIN (GALACTOARABINO-RHAMNOGALACTURONATE, GR-MD-02)

Belapectin is a galectin-3 antagonist developed by Galectin (Rotman and Sanyal, 2017; Neuschwander-Tetri, 2020). Early preclinical studies have shown that Belapectin is a candidate drug for anti-fibrosis research and can reverse the degree of hepatic fibrosis in steatohepatitis mice and prevent collagen deposition before the occurrence of fibrotic cells (**Figures 2**, **3**) (Rotman and Sanyal, 2017; Iacobini et al., 2011). Elevated galactosin-3 levels are associated with NASH and induce hepatic fibrosis in mice (Iacobini et al., 2011; Harrison et al., 2016). Belapectin, an inhibitor of galactosin-3, can alleviate hepatic fibrosis and portal hypertension in rats and was shown to be safe and well-tolerated in a phase I trial (NCT01899859) (Harrison et al., 2016). Galectin is currently in a phase III trial to evaluate Belapectin for the prevention and treatment of nonalcoholic fatty liver disease (NAFLD), portal hypertension, fibrosis, psoriasis, liver function decline, and other diseases (Chalasani et al., 2020).

Belapectin single and three weekly repeated at 2, 4, and 8 mg/kg demonstrated no meaningful clinical differences in treatment-emergent adverse events, vital signs, electrocardiographic findings, or laboratory tests. Pharmacokinetic parameters showed a dose-dependent relationship, with evidence of drug accumulation following 8 mg/kg (Harrison et al., 2016). Results of a 52-weeks phase IIb trial suggest that Belapectin is an effective anti-fibrotic drug for compensatory NASH cirrhosis (NCT02462967) (Chalasani et al., 2020). Because the treatment can take many years, there is an urgent need for effective drug candidates with good safety and tolerability (NCT02421094) (Harrison et al., 2018b). Overall, the clinical success of Belapectin indicates a promising path for the

continued clinical development of Belapectin in compensatory NASH cirrhosis, which could make it the first anti-fibrosis drug candidate to win approval from NASH regulators (Chalasani et al., 2018).

CENICRIVIROC (TAK-652, TBR-652)

Cenicriviroc is a chemokine receptor 2/5 (CCR2/5) antagonist that acts differently from previous anti-HIV1 drugs and holds great promise in the field of anti-AIDS drugs (**Figure 1**) (Friedman et al., 2016; Friedman et al., 2018). The formula of Cenicriviroc is $C_{41}H_{52}N_4O_4S$, with a molecular weight of 696.94. Studies have found that monocyte chemokine protein-1 (MCP-1), chemokine C-C motif chemokine 2 (CCL2), and regulated upon activation, normal T cells expressed and secreted (RANTES) can promote the aggregation of monocytes/macrophages in blood to liver inflammatory sites through CCR2/5, as well as produce various cytokines, such as transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, to further activate HSCs and generate ECM, which leads to the formation of hepatic fibrosis (NCT02217475) (Friedman et al., 2016; Lefebvre et al., 2016a; Lefebvre et al., 2016b). Additionally, CCR2 and CCR5 are highly expressed in activated HSCs, which directly or indirectly mediate various biological functions of HSCs after binding with its ligand and participate in the formation of hepatic fibrosis (NCT02217475) (Lefebvre et al., 2016a; Lefebvre et al., 2016b). Therefore, CCR2 and CCR5 have become important targets for anti-fibrosis therapy (**Figures 2**, **3**).

Cenicriviroc is a novel oral drug developed in collaboration between Takeda and Tobira Therapeutics. The plasma half-life of Cenicriviroc is 30–40 h, and it can be administered once a day (NCT02217475) (Lefebvre et al., 2016a; Friedman et al., 2016). Cenicriviroc has shown good safety, providing new ideas and methods for the clinical prevention and treatment of hepatic fibrosis (Lefebvre et al., 2016a; Friedman et al., 2016). Animal experiments have shown that Cenicriviroc significantly alleviates thioacetamide (TAA)-induced hepatic fibrosis in rats, mainly by inhibiting HSC synthesis of collagen I, thereby inhibiting collagen deposition in the liver and reducing liver tissue inflammation (Lefebvre et al., 2016b; Kruger et al., 2018). Therefore, Cenicriviroc may represent an effective drug against hepatic fibrosis.

Clinical studies have found that Cenicriviroc has excellent pharmacokinetic properties in the human body and is well tolerated without causing dose-limiting adverse reactions (Friedman et al., 2016; Friedman et al., 2018). Cenicriviroc is also well absorbed and slowly eliminated (Anstee et al., 2020; Reimer et al., 2020). Additionally, the majority of adverse reactions were mild (grade 1 or 2) and dose-independent, with gastrointestinal disturbances (63%) and systemic adverse reactions (37%) being the most common. Grade 3 adverse events (abscesses) were reported by one subject in each of the placebo and 75 mg (qd) groups, but they were not considered to be related to the product. There were no grade 4 adverse reactions, severe adverse reactions, death, or withdrawal from the study due to adverse reactions (Sumida and Yoneda, 2018; Sumida et al., 2019). A phase II trial showed that Cenicriviroc rapidly blocks CCR2 and CCR5 (Sumida and Yoneda, 2018; Sumida et al., 2019), and it has been shown that 150 mg Cenicriviroc can be used to treat mild and moderate liver injury, with good tolerance. The pharmacokinetic data of Cenicriviroc is relatively complete and it represents a promising drug for treating hepatic fibrosis (Lefebvre et al., 2016a). Cenicriviroc is currently in a phase III trial (NCT03028740) (Sumida and Yoneda, 2018; Pedrosa et al., 2020).

ELAFIBRANOR (GFT505)

Elafibranor is a double agonist of peroxisome proliferator-activated receptors α/δ (PPARα/δ) developed by Genfit, France; its molecular formula is $C_{22}H_{24}O_4S$, and its molecular weight is 384.489 (Boeckmans et al., 2019; Cheng et al., 2019). The chemical structure is shown in **Figure 1**. Elafibranor alleviates NASH symptoms through various mechanisms, including increased fatty acid oxidation, improved lipid profile, increased insulin sensitivity, and anti-inflammatory and anti-fibrosis effects (**Figures 2**, **3**) (Alukal and Thuluvath, 2019; Ratziu et al., 2019).

Animal experiments have shown that Elafibranor administration can effectively reduce hepatic steatosis, inflammation, fibrosis, and the level of liver dysfunction biomarkers, as well as inhibit the expression of pro-inflammatory and pro-fibrosis genes; it is effective in both the prevention and treatment of hepatic fibrosis (Schuppan et al., 2018; Baandrup Kristiansen et al., 2019; Roth et al., 2019). Elafibranor treatment protects against hepatic steatosis and inflammatory progression (Ratziu et al., 2016; Baandrup Kristiansen et al., 2019), and has shown preventive and therapeutic effects on CCl_4-induced hepatic fibrosis in rats (Schuppan et al., 2018). In pharmacokinetic studies conducted in rats, Elafibranor and its metabolites were rapidly excreted into bile and underwent extensive enterohepatic circulation (Schuppan et al., 2018). High concentrations of Elafibranor and/or its metabolites were detected in the liver and intestines, with little distribution in other tissues (Schuppan et al., 2018; Roth et al., 2019).

No toxicity or carcinogenesis of Elafibranor was found in long-term animal toxicity tests (Roth et al., 2019; Gore et al., 2020). From 2011 to 2015, Genfit completed a series of phase I clinical trials of Elafibranor, which were found to be both safe and well-tolerated. The safety of Elafibranor is generally good in many clinical trials, and no serious adverse events have occurred to date (Boeckmans et al., 2019; Cheng et al., 2019; Alukal et al., 2016). In completed phase II clinical trials, Elafibranor has shown a certain therapeutic efficacy and good safety and tolerability (Ratziu et al., 2016; Alukal and Thuluvath, 2019). Fast-track approval was granted by the FDA in February 2014, and a phase III clinical trial began in March 2016 (Ratziu et al., 2016; Alukal and Thuluvath, 2019). In January 2018, Genfit announced that the FDA had approved Elafibranor for the pediatric study program and would begin a clinical trial for the treatment of pediatric NASH. Elafibranor is currently in phase III of the clinical trial,

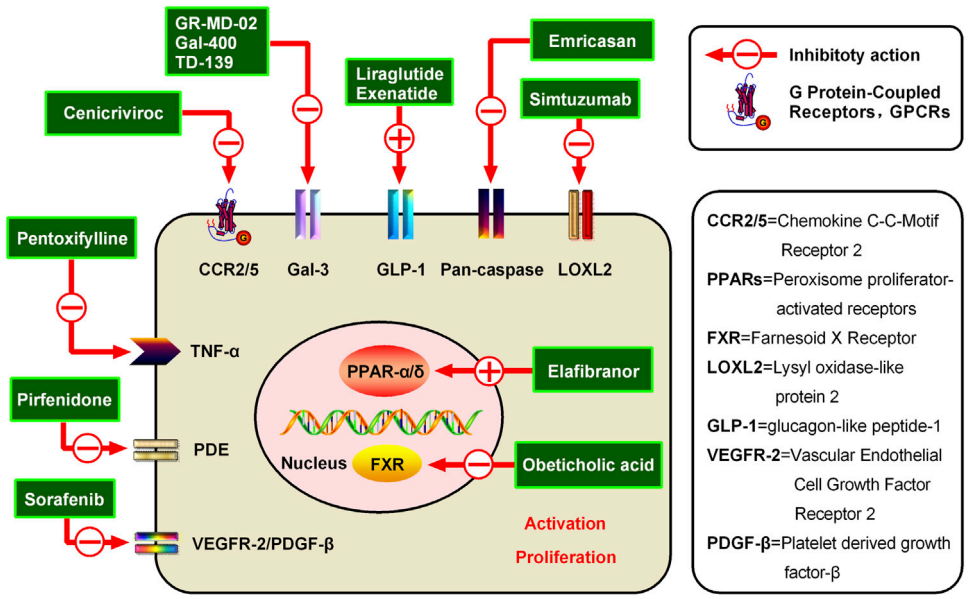

FIGURE 3 | Potential candidates for hepatic fibrosis and their mechanisms of action. Inhibiting the activation and proliferation of HSCs is an important method for the prevention and treatment of hepatic fibrosis. Various representative drugs have emerged for different targets. (1) PPARs are members of the nuclear receptor superfamily, and the PPAR agonist Elafibranor can inhibit liver fibrosis through direct anti-inflammatory effects and indirect improvement of the oxidative stress state. (2) Additionally, a representative FXR receptor agonist, Obeticholic acid, has been shown to reduce liver inflammation and promote ECM degradation to alleviate hepatic fibrosis. (3) Simtuzumab is a monoclonal antibody currently being developed by Gilead for NASH, cirrhosis, and advanced hepatic fibrosis blocking of LOXL2. LOXL2 is a protease that modifies the ECM by promoting the cross-linking of collagen fibers and is believed to play an important role in tumor progression and fibrosis. (4) Emricasan is an irreversible pan-caspase inhibitor, which can reduce the activity of caspases to improve the inflammatory environment and inhibit HSC activation. (5) The GLP-1R agonist Liraglutide can inhibit the formation of ECM and reduce the liver inflammatory response and fibrosis process. (6) Belapectin is a galectin-3 antagonist, which can alleviate hepatic fibrosis and portal hypertension in rats and was found to be safe and well-tolerated in a phase I trial. (7) The CCR2/5 antagonist Cenicriviroc can improve hepatic fibrosis by inhibiting liver inflammation. Cenicriviroc is not only effective for early hepatic fibrosis but also feasible for maintenance treatment in patients with advanced fibrosis. (8) Pentoxifylline is a non-specific PDE inhibitor, which can increase intracellular cAMP concentration and plays an anti-hepatic fibrosis role by inhibiting TNF-α production. (9) Attenuating TGF-β-induced signal transduction pathways can inhibit hepatic fibrosis, such as via Pirfenidone (10) Sorafenib can inhibit VEGFR-2 and PDGF-β to alleviate hepatic fibrosis.

and subgroup analyses of results support its potential efficacy in patients with severe NASH (Boeckmans et al., 2019; Cheng et al., 2019). Currently, there is no approved effective drug for NASH (Alukal and Thuluvath, 2019; Boeckmans et al., 2019). Of nearly 200 candidates in development worldwide, Elafibranor is one of the most highly anticipated drugs for NASH to hit the market.

In a 52-weeks phase II study in patients with NASH without cirrhosis, 276 patients were randomly assigned to receive Elafibranor at either 80 mg or 120 mg daily or a placebo. Even though the trial was not designed with antifibrotic goals, it is worth noting that among the patients who responded to 120 mg Elafibranor (n = 17), there was a reduction in fibrosis ($p < 0.001$) compared to non-responders to the same regimen. However, at present, there are limited pharmacokinetic data on this drug in the human body, which needs further study (Ratziu et al., 2016). On 11 May 2020, Genfit announced that in its phase III trial of Elafibranor in the treatment of NASH, the drug failed to significantly improve patients' NASH histological symptoms, and in some cases, exacerbated hepatic fibrosis compared to placebo (Guaraldi et al., 2020; Shen and Lu, 2021). A total of 1070 patients with NASH were randomized (2:1) to receive Elafibranor 120 mg/day or a placebo (Guaraldi et al., 2020; Shen and Lu, 2021). After 72 weeks, 19.2% and 14.7% of patients treated with Elafibranor and placebo had improved NASH histology without worsening hepatic fibrosis, respectively, 24.5% and 22.4% of patients with at least a grade of fibrosis improvement, however, these results were not statistically significant. The safety of Elafibranor was consistent with the results of previous studies (Guaraldi et al., 2020; Shen and Lu, 2021) in that no adverse effects, such as increased fluid retention and heart failure, were mediated by PPARγ (Guaraldi et al., 2020; Shen and Lu, 2021).

EMRICASAN (IDN-6556, PF-03491390)

Emricasan is a pioneering and irreversible pan-caspase inhibitor that is orally administered (**Figure 1**) (Garcia-Tsao et al., 2019; Barreyro et al., 2015). The formula of Emricasan is $C_{26}H_{27}F_4N_3O_7$, and its molecular weight is 569.50. Emricasan can be retained in the liver for a long time and can reduce the activity of caspases, which mediate inflammation, cell death, and apoptosis (Garcia-Tsao et al., 2019; Barreyro et al., 2015; Gracia-Sancho et al., 2019). By reducing the activity of these enzymes, Emricasan may block the development of liver disease (Gracia-Sancho et al., 2019; Shiffman et al., 2019). Emricasan, developed

by Conatus, has been shown in preclinical studies to reduce apoptosis, improve the inflammatory environment and inhibit HSC activation (**Figures 2**, **3**) (Barreyro et al., 2015; Gracia-Sancho et al., 2019; Shiffman et al., 2019).

Emricasan reduced liver damage in NASH but had no significant effect on metabolic disorders (Frenette et al., 2019; Harrison et al., 2020). Emricasan was previously demonstrated to inhibit some of the liver enzymes which lead to liver inflammation and fibrosis (Frenette et al., 2019; Harrison et al., 2020). In a mouse model of NASH, treatment with Emricasan attenuated hepatic fibrosis and the activation of HSCs (Barreyro et al., 2015; Gracia-Sancho et al., 2019). Emricasan is currently in a phase II clinical trial to test its efficacy in treating liver injury and fibrosis through chronic HCV infection (Frenette et al., 2019). In two previous trials, Emricasan was both well-tolerated and safe, and the results were consistent with those of 19 previous clinical studies (Frenette et al., 2019; Shiffman et al., 2019). Emricasan treatment was safe and well-tolerated, with adverse events, severe events, abnormal experimental results, vital signs, cancers, and infections occurring at similarly low rates in the Emricasan treatment group and placebo groups (Frenette et al., 2019; Shiffman et al., 2019; Harrison et al., 2020). The most common adverse reactions in the Emricasan group were headache (16%), nausea (14%), and fatigue (9%) (Frenette et al., 2019; Shiffman et al., 2019; Harrison et al., 2020). Results from a multicenter phase b clinical trial suggest that Emricasan improves liver function in patients with severe cirrhosis (NCT02686762) (Garcia-Tsao et al., 2020). Moreover, a 28-days randomized clinical trial of Emricasan assessed the efficacy, safety, and tolerability of Emricasan in subjects with NAFLD, in which the subjects were randomized to Emricasan 25 mg twice daily or a matching placebo. The results showed that Emricasan decreased ALT and biomarkers in subjects with NAFLD and raised AST after 28 days (NCT02077374). Emricassan has a high first-pass metabolism, but the current pharmacokinetic data are mainly from animal experiments, with a lack of complete human experimental data (Garcia-Tsao et al., 2019).

LIRAGLUTIDE (VICTOZA)

Liraglutide is a Glucagon-Like Peptide (GLP-1) analog developed by Novo Nordisk to decrease blood sugar in patients with type 2 diabetes mellitus (T2DM) (**Figure 1**) (Kahal et al., 2014; Zoubek et al., 2017). The formula of Liraglutide is $C_{172}H_{265}N_{43}O_{51}$, and its molecular weight is 3751.26. GLP-1 can increase insulin release, decrease glucagon secretion, reduce hepatic steatosis, and improve hepatic fibrosis (Fagone et al., 2016; Briand et al., 2020). GLP-1R agonists can reduce liver cell apoptosis and endoplasmic reticulum stress through various mechanisms. Previuous studies of mechanisms have been proposed, including an increase in cyclic adenosine monophosphate (cAMP) production, activation of an AMP-activated protein kinase (AMPK)-dependent pathway in hepatocytes increasing fatty acid oxidation and decrease in lipogenesis, and/or an increase in hepatic insulin signaling and sensitivity with GLP-1 and subsequent improvement of the hepatic glucose metabolism. Additionally, Liraglutide has been found to reduce fatty acid accumulation, in mice fed a high-fat diet by enhancing autophagy and reducing endoplasmic reticulum stress-related apoptosis. Furthermore, the GLP-1R agonist can reduce liver cell apoptosis and endoplasmic reticulum stress by inhibiting activation of the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome and NF-κB signaling pathway. Besides, Liraglutide can promote liver glucose and lipid metabolism, and inhibit the secretion of inflammatory cytokines, which may explain its ability to alleviate the process of hepatic fibrosis (Kahal et al., 2014; Zoubek et al., 2017; Fagone et al., 2016). Studies have also shown that Liraglutide can inhibit the formation of ECM, reduce the liver inflammatory response and fibrosis process, and slow and improve the progression of NAFLD in patients with T2DM (**Figures 2**, **3**) (Kahal et al., 2014; Fagone et al., 2016; Zoubek et al., 2017).

The results of animal experiments showed that Liraglutide could significantly reduce collagen fibers in NAFLD models (Fagone et al., 2016; Choi et al., 2019; Briand et al., 2020). Moreover, Liraglutide significantly reduced levels of inflammatory factors, such as IL-6 and TNF-α, and liver fibrosis factors in the NAFLD model group (Fagone et al., 2016; Choi et al., 2019; Briand et al., 2020). Liraglutide may play an important role in NAFLD by activating the SIRT1/AMPK pathway, regulating key regulatory molecules of lipid synthesis and metabolism, and inhibiting the *de novo* synthesis of fatty acids (Kahal et al., 2014). Early intervention with Liraglutide can reduce blood glucose, inhibit fat synthesis, reduce insulin resistance, inflammation, fibrosis, and oxidative stress damage, thereby alleviating NAFLD, which may be related to the activation of SIRT1/AMPK and its downstream genes (Kahal et al., 2014). These results indicate that Liraglutide may represent a potential drug for the treatment of NAFLD, with SIRT1 as a potential therapeutic target.

Recently, in addition to its good hypoglycemic effect, Liraglutide has also been shown to inhibit myocardial fibrosis, renal fibrosis, and hepatic fibrosis (Kahal et al., 2014). Current studies suggest that inflammation is the core cause of hepatic fibrosis, which is mainly associated with NF-κB activation, and initiation of the NF-κB signaling pathway (Gilgenkrantz et al., 2021). Therefore, inhibiting NF-κB mitigates various inflammatory liver diseases, including hepatic fibrosis. Liraglutide may play anti-inflammatory and anti-fibrosis roles by inhibiting the activation of NF-κB and reducing the production of superoxide (Fagone et al., 2016). Liraglutide can also reduce the degree of tissue collagen deposition and improve tissue fibrosis (Armstrong and Newsome, 2015; Choi et al., 2019; Briand et al., 2020), and may play an anti-fibrosis role by regulating some important links in the fibrotic process (Armstrong et al., 2015; Armstrong et al., 2016; Gaborit et al., 2016; Choi et al., 2019). The data from genetic toxicity studies show that Liraglutide is not toxic in the human body (Armstrong et al., 2015; Armstrong et al., 2016; Gaborit et al., 2016). Results of a phase II multicentered trial showed improvement in NASH and no further increase in hepatic fibrosis in 39% of participants in the

treatment group compared to only 9% in the placebo group (NCT02654665). Pharmacokinetic studies have shown that about 99% of Liraglutide binds to albumin in the body, allowing it to escape glomerular filtration and extend its duration of action (Alruwaili et al., 2021). It is expected to be one of the candidate drugs for the treatment of hepatic fibrosis in the future.

OBETICHOLIC ACID (INT-747, 6-ECDCA, 6A-ETHYLCHENODEOXYCHOLIC ACID)

Farnesoid X Receptor (FXR) is a member of the nuclear receptor superfamily and is classified as NR1H4 (**Figure 1**) (Eslam et al., 2019; Anfuso et al., 2020). FXR is mainly expressed in the enterohepatic system, FXRα1/2 and FXRα3/4 are expressed in the liver, while FXRα3/4 is mainly expressed in the intestine. FXR is widely involved in the pathophysiological processes of many diseases in the enterohepatic system (Armstrong et al., 2015; Gawrieh et al., 2019). Activated FXR plays a protective role in various chronic liver diseases (Brunt et al., 2019; Eaton et al., 2020), and FXR agonists include Obecholic Acid, GW4064, WAY-362450, PR20606, GS9674, and LJN452, which are a batch of synthetic or semi-synthetic FXR agonists. Among them, GW4064 and Obecholic Acid are widely used. The formula of Obecholic Acid is $C_{26}H_{44}O_4$, and its molecular weight is 420.63. GW4064 is a synthetic nonsteroidal FXR agonist (**Figures 2**, **3**).

FXR has been found to be associated with hepatic fibrosis, cirrhosis, and portal hypertension (Younossi et al., 2019; Hindson, 2020; Siddiqui et al., 2020). The activation of HSCs is a key factor in the development of hepatic fibrosis, and it has been previously reported that FXR activation mitigates hepatic inflammation. One mechanism by which FXR reduces inflammatory mediator expression in HSCs is via the induction of PPARγ. Additionally, FXR activation represses gluconeogenesis, TG synthesis, and VLDL export via SHP, a primary FXR-responsive gene, which contributes to regulating FXR target genes. FXR modulates many genes by regulating SHP. Besides, regulation of the MMP/TIMP balance is essential for the transition of the physiological ECM into pathological ECM. The FXR agonist Obecholic Acid has been shown to induce MMP2–9 activity and a dose-dependent reduction of collagen. Therefore, FXR inhibits HSC activation by activating the PPARγ and SHP-TIMP pathways. Additionally, FXR improves portal hypertension by increasing eNOs activity and reducing vascular remodeling (Anfuso et al., 2020). These findings, together with the regulation of metabolic and inflammatory functions, strongly suggest that FXR is an ideal target for the treatment of hepatic fibrosis, cirrhosis, and portal hypertension (Brunt et al., 2019; Gawrieh et al., 2019; Younossi et al., 2019). Studies have shown that the FXR agonist Obecholic Acid can reduce liver inflammation and fibrosis in TAA-induced toxic cirrhosis, and even reverse hepatic fibrosis (Anfuso et al., 2020). FXR inhibits the negative regulator NF-κB by up-regulating IκBα, resulting in reduced expression of pro-fibrotic cytokines and related markers of hepatocyte transformation, thereby weakening the effect of inflammatory cytokines (Younossi et al., 2019).

Hepatic fibrosis is caused by persistent liver injury and is characterized by inflammation, activation of HSCs, accumulation of ECM, and destruction of the liver structure. HSCs proliferate and transform into myofibroblasts, which deposit collagen I and fibronectin in the ECM and eventually produce fibrogenic cytokines (Brunt et al., 2019; Eslam et al., 2019; Anfuso et al., 2020). FXR prevents the activation of HSCs through FXR-PPARγ or FXR-SHP-TIMP pathways. In animal models of hepatic fibrosis induced by TAA, bile duct ligation (BDL), and CCl_4, Obecholic Acid significantly reduced the expression of hepatic fibrosis genes and proteins, as well as the area of hepatic fibrosis parenchymal tissue (Eslam et al., 2019; Anfuso et al., 2020). The FXR agonist Obecholic Acid has also been shown to increase the interaction between FXR and Smad3 and alleviate CCl_4-induced liver injury and fibrosis. Studies have shown that FXR gene knockout mice can increase liver inflammation and fibrosis, suggesting that the loss of FXR function is more likely to induce liver inflammation and fibrosis. Clinical trials also showed that Obecholic Acid significantly improves hepatic fibrosis, ballooning degeneration, steatosis, and lobular infiltration (Brunt et al., 2019; Younossi et al., 2019). In addition to Obecholic Acid, other FXR agonists such as PX-102, Way-362450, and EDP-305 have been shown to have protective effects against diet-induced fibrosis such as MCD (Anfuso et al., 2020). Taken together, these preclinical studies support the anti-fibrosis effects of FXR agonists.

The FXR agonist obecholangitis was approved by the FDA on 27 May 2016, for treating primary biliary cholangitis (PBC) in adults (Eaton et al., 2020). Results from two phase II clinical trials showed that Obecholic Acid significantly improved the disease activity scores in patients with NAFLD, as well as steatosis, lobular inflammation, ballooning degeneration, and hepatic fibrosis (Younossi et al., 2019; Anfuso et al., 2020). The results of a phase II clinical trial (NCT00501592) showed that Obeticholic Acid significantly improved insulin sensitivity in patients with T2DM complicated with NAFLD, significantly improved hepatic fibrosis, and liver enzymology indexes ($p < 0.05$). Additionally, there were no adverse reactions during the trial, and both safety and tolerability were good. However, elevated serum alkaline phosphatase and total cholesterol concentrations in patients during the study should be seriously considered.

Recently, a phase III clinical trial of Obecholic Acid in the treatment of NASH with fibrosis has been completed (Younossi et al., 2019; Hindson, 2020). In the trial, 18% of patients who received 10 mg Obecholic Acid and 23% who received 25 mg Obecholic Acid showed improvement in hepatic fibrosis. Another double-blind placebo-controlled trial investigating the efficacy and safety of Obecholic Acid in the treatment of type 2 diabetes complicated by NAFLD also demonstrated a significant reduction in hepatic fibrosis markers in patients taking 25 mg Obecholic Acid (Younossi et al., 2019; Hindson, 2020). These clinical studies demonstrate that the FXR agonist Obecholic Acid is promising for the treatment of hepatic fibrosis. Recently, some scholars have suggested that specific micro RNA is also a target gene of FXR, which can regulate the process of hepatic fibrosis. After mice and human HSCs were treated with the FXR agonist GW4064, miR-

29a levels increased, ECM accumulation decreased, and hepatic fibrosis was alleviated. Additionally, the level of FXR in liver tissue of patients with severe hepatic fibrosis was decreased and the level of miR-199a-3p was increased, while the expression of miR-199a-3p was inhibited after activation of FXR, which further inhibited the proliferation of HSCs and alleviated hepatic fibrosis.

Since Obecholic Acid was marketed in May 2016, 19 deaths have been confirmed, of which 8 have provided the cause of death information and seven patients with moderate to severe liver dysfunction may have been caused by the use of the drug beyond the recommended dose (Younossi et al., 2019). The FDA recommends that physicians determine a patient's baseline liver function before administering Obecholic Acid and strictly adhere to the approved dosing regimen (Younossi et al., 2019; Siddiqui et al., 2020). The most common adverse reactions shown in clinical trials were severe pruritus, which resulted in discontinued treatment in some patients at higher doses. Some patients also experienced fatigue, abdominal pain and discomfort, arthralgia, constipation, elevated blood sugar, elevated blood lipids, dizziness, and dysarthria, which may be caused by cerebral ischemia. The observed increase in LDL and decrease in HDL suggest that this drug may lead to the occurrence of cardiovascular and cerebrovascular events (Eslam et al., 2019; Younossi et al., 2019; Siddiqui et al., 2020). However, these adverse reactions can be effectively alleviated after dose control and medication regimen adjustment. Obecholic Acid is subject to enterohepatic circulation, and the pharmacokinetic parameters of the active metabolites show that food may increase its absorption (Wang et al., 2021).

PENTOXIFYLLINE

Pentoxifylline, a derivative of Methylxanthine, is a non-specific phosphodiesterase inhibitor developed by Pharm Holdings, with various pharmacological characteristics, mainly used in cerebrovascular diseases (**Figure 1**) (Oberti et al., 1997; Desmoulière et al., 1999; Chooklin and Perejaslov, 2003). The formula of Methylxanthine is $C_{13}H_{18}N_4O_3$, and its molecular weight is 278.31. Recent studies have found that Pentoxifylline also has a strong anti-fibrosis effect, which can effectively inhibit hepatic fibrosis, kidney fibrosis, and skin scar formation (Peterson, 1993; Pinzani et al., 1996; Verma-Gandhu et al., 2007). Pentoxobromine is a first-line drug for the treatment of AH, which has anti-inflammatory, anti-hepatic fibrosis, and immunological regulation effects (Louvet et al., 2008; Ali et al., 2018). However, the specific mechanism of Pentoxifylline is still unclear (**Figures 2**, **3**).

The Hedgehog signaling pathway plays an important role in cell differentiation and proliferation during embryonic development. Recent studies have found that the hedgehog signaling pathway is involved in the repair of liver injury and the occurrence of hepatic fibrosis (Solhi et al., 2022). Furthermore, Pentoxifylline may block the activation of HSCs by inhibiting the hedgehog signaling pathway and inhibit the occurrence of hepatic fibrosis in Schistosoma, and is therefore expected to be an effective drug for the prevention and treatment of Schistosoma in clinical practice. Additionally, PPAR-α and NF-κB P65 have been studied in many fields and are closely related to AH due to their anti-inflammatory, anti-oxidation, and anti-hepatic fibrosis effects, and regulation of fat metabolism (Ali et al., 2018). Current studies have found that Pentoxifylline can treat AH in rats, and its mechanism may be related to the upregulation of PPAR-α expression and downregulation of NF-κB P65 expression (Satapathy et al., 2007; Ali et al., 2018).

Through non-selective inhibition of phosphodiesterase, Pentoxifylline reduces intracellular cAMP hydrolysis to 5-AMP and increases intracellular cAMP concentrations, inducing corresponding changes in cells and inhibition of the calcium ion influx (Pinzani et al., 1996). Pentoxifylline improves hemorheology in many complementary ways, including reducing blood and plasma viscosity, reducing plasma fibrinogen, and promoting fibrinolysis. Additionally, Pentoxifylline improves blood permeability in tissues by enhancing the plasticity of erythrocytes and reducing neutrophil activation. Pentoxifylline has a protective effect on the liver by improving liver hemorheology and has anti-inflammatory and anti-fibrosis effects (Zein et al., 2012; Park et al., 2014). As an anti-inflammatory and anti-fibrosis drug, Pentoxifylline can inhibit the production of TNF-α, a pro-inflammatory cytokine, at 400 mg/day, 3 times per day for 28 days, which can effectively delay the disease progression in patients with severe alcoholic liver disease. Additionally, the anti-fibrosis and anti-inflammatory effects of Pentoxifylline have certain protective effects on the liver. The mechanism of action of Pentoxifylline is completely different from that of other commonly used liver protective drugs, so a combination of Pentoxifylline can produce complementary effects (Lebrec et al., 2010; Zein et al., 2012; Park et al., 2014).

As a non-specific phosphodiesterase inhibitor, Pentoxifylline has anti-inflammatory and anti-fibrosis effects, improves hepatic hemorheology, inhibits hyperplasia, and has certain effects on the prevention and treatment of cirrhosis and reduces mortality of liver disease (Ali et al., 2018). Existing clinical evidence shows that Pentoxifylline combined with corticosteroids can reduce the risk of death in severe alcoholic hepatitis (Louvet et al., 2008). Pentoxifylline can improve the histological characteristics of NAFLD/NASH, prevent hepatic steatosis, and improve liver function in patients with non-dyslipidemia NAFLD (Satapathy et al., 2007; Zein et al., 2012). Furthermore, Pentoxifylline can improve the survival rate of patients with HRS and is expected to be a therapeutic drug for HRS. Pentoxifylline hit the market earlier, and its efficacy, toxicity, and pharmacokinetic data are relatively complete. Previous studies have found that Pentoxifylline can be rapidly and widely absorbed from the gastrointestinal tract of animals and humans and rapidly metabolized systemically (Ward and Clissold, 1987).

PIRFENIDONE

Pirfenidone, chemically known as 5-Methyl-N-phenyl-2-1H-pyridone, is a new kind of pyridinone compound with a broad spectrum of anti-fibrosis effects, which can prevent and reverse

the formation of fibrosis and scars (**Figure 1**) (Grizzi, 2009; Di Sario et al., 2002). The formula of Pirfenidone is $C_{12}H_{11}NO$, and its molecular weight is 185.22. Pirfenidone, marketed by Shionogi in 2008 and approved by the FDA, is the first drug to demonstrate some efficacy for idiopathic pulmonary fibrosis (IPF) in a repeated, randomized, placebo-controlled phase III clinical trial (Armendáriz-Borunda et al., 2006; Verma et al., 2017; Sandoval-Rodriguez et al., 2020). Moreover, it also has a good effect on fibrosis diseases, such as renal interstitial fibrosis and hepatic fibrosis. However, its mechanism of action for treating IPF is still unclear (Benesic et al., 2019; Zhang et al., 2019). Current studies have shown that Pirfenidone can reduce the proliferation of lung fibroblasts and their differentiation into myofibroblasts by attenuating TGF-β-induced signal transduction pathways (Smad3, P38, and Akt), decreasing the expression of recombinant Heat Shock Protein 47 (HSP47) induced by TGF-β, reducing the expression of α-SMA and collagen I (**Figures 2**, **3**) (Di Sario et al., 2002; Benesic et al., 2019; Sandoval-Rodriguez et al., 2020).

Pirfenidone can inhibit the proliferation of hepatocytes and promote hepatocyte apoptosis by inhibiting the Wnt/β-Catenin signaling pathway, which is an effective oral small-molecule drug for the treatment of fibrosis. Many studies have found that Pirfenidone has important anti-inflammatory and anti-fibrosis effects *in vivo* and *in vitro* (Di Sario et al., 2002; Grizzi, 2009; Benesic et al., 2019). Pirfenidone can regulate TGF-β and TNF-α and inhibit fibroblast proliferation and collagen synthesis (García et al., 2002; Salah et al., 2019; Ullah et al., 2019). Moreover, Pirfenidone can significantly alleviate hepatic fibrosis induced by CCl_4 in mice; this effect is closely related to the mechanism of reducing expression levels of PI3K and PKB in the mouse liver (Grizzi, 2009; Benesic et al., 2019). These results suggest that Pirfenidone is a potential therapeutic agent for hepatic fibrosis.

Of the 978 adverse events reported, 17% were nausea and vomiting, 16% were diarrhea, 10% were fatigue, 9% were loss of appetite, 6% were dyspepsia, 6% were rash (n = 59), 5% were headache, and 10% were others (Maher et al., 2020). The incidence of other adverse events was lower than 5%; the most common was acute respiratory failure (2 cases), indicating that the disease is progressive (Maher et al., 2020). Overall, these data suggest that long-term oral administration of Pirfenidone does not increase the risk of ADRs, which is consistent with the known safety characteristics of Pirfenidone. Pirfenidone is a small synthetic molecule with high oral bioavailability, which is primarily metabolized via the liver, although the specific mode of metabolism remains unknown. Currently, the pharmacokinetic data obtained are from trials with small sample sizes, and large-scale clinical trial data are still needed (Cho and Kopp, 2010).

SIMTUZUMAB (GS-6624, INN, SIM)

Simtuzumab is a monoclonal antibody that targets blocking Lysyl Oxidase Like Protein-2 (LOXL2) (Harrison et al., 2018a; Fickert, 2019). LOXL2 is a protease that modifies the ECM by promoting cross-linking of collagen fibers, is believed to play an important role in tumor progression and fibrosis, with the potential to inhibit tumor progression and reverse fibrosis (**Figures 2**, **3**) (Muir et al., 2019; Sanyal et al., 2019).

It has been reported that LOXL2 can promote the occurrence of liver cell fibrosis by catalyzing collagen cross-linking (Meissner et al., 2016; Raghu et al., 2017). Researchers have investigated the safety and efficacy of Simtuzumab in patients with advanced fibrosis caused by NASH (Meissner et al., 2016; Sanyal et al., 2019). In a double-blind study of 219 patients with bridging fibrosis caused by NASH, the patients were randomly assigned (in a 1:1:1 ratio) to a subcutaneous injection of Simtuzumab (75 or 125 mg) or placebo each week for 240 weeks. The experiment was stopped after week 96, and the results showed that liver collagen levels were significantly reduced in all three groups of patients with bridging fibrosis, including those who were given a placebo (Sanyal et al., 2019).

Gilead designed five phase II trials for Simtuzumab to investigate the potential of Simtuzumab in the treatment of pancreatic cancer, colorectal cancer, myeloid fibrosis, IPF, and hepatic fibrosis (Harrison et al., 2018a; Fickert, 2019). To date, two phase II trials of Simtuzumab have failed in pancreatic cancer and colorectal cancer, the remaining three remain to be observed. The latter two indications (pulmonary fibrosis and hepaitc fibrosis) may be the best bet for Simtuzumab. Gilead is currently investigating the efficacy of Simtuzumab for treating IPF (Fickert, 2019; Muir et al., 2019; Sanyal et al., 2019).

A multicenter phase II trial evaluated the efficacy and safety of Selonsertib (a selective inhibitor of ASK1) or in combination with Simtuzumab in patients with NASH and stage 2 or 3 hepatic fibrosis (Harrison et al., 2018b). A total of 72 patients were randomized to open treatment for 24 weeks. Patients were treated with 6 mg or 18 mg Selonsertib orally once daily along with or without a weekly injection of 125 mg Simtuzumab, or with Simtuzumab alone. The results showed that hepatic fibrosis improved in 20% of patients treated with Simtuzumab alone after 24 weeks of treatment. The improvement of hepatic fibrosis was related to decreased liver hardness, reduced collagen content and inflammation of liver lobules, as well as an improvement in serum biomarkers of apoptosis and necrosis (Harrison et al., 2018a). There were no significant differences in side effects among the three treatment groups. At present, no pharmacodynamic and pharmacokinetic assays are available to assess whether LOXL2 is indeed effectively inhibited in the human liver, and further studies are needed (Fickert, 2019).

SORAFENIB (BAY43-9006, NEXAVAR)

Sorafenib is a small molecule compound whose chemical name is 4-[4-({[4-chloro-3-(trifluoromethyl) phenyl] carbamoyl} amino) phenoxy]-Nmethylpyridine-2-carboxamide (**Figure 1**) (Thabut et al., 2011; Liu et al., 2018), the formula is $C_{21}H_{16}ClF_3N_4O_3$, and the molecular weight is 464.82. Sorafenib is an oral multiple kinase inhibitor and a novel multi-molecular target chemotherapy drug. Sorafenib mainly functions by inhibiting tumor cell proliferation, inhibiting angiogenesis, and promoting tumor cell apoptosis (Chen et al., 2014; Deng et al., 2013). Clinically, Sorafenib is mostly used for the treatment of advanced malignant tumors, particularly liver cancer (Lin et al., 2016; Faivre et al., 2020).

Sorafenib can prolong the survival of patients with advanced HCC by 3 months on average, but due to congenital or acquired resistance of patients, it is usually not longer than 6 months before Sorafenib resistance occurs (**Figures 2**, **3**) (Faivre et al., 2020).

Hepatic fibrosis, as an important feature of the pathogenesis of chronic liver diseases, has always been one of the important topics in the field of liver disease research and treatment (Ma et al., 2017; Sung et al., 2018). The causes of hepatic fibrosis are complex, TGF-β is the most important known factor promoting hepatic fibrosis (Deng et al., 2013; Ma et al., 2017; Faivre et al., 2020). Hepatic parenchymal cells secrete TGF-β during injury and inflammation, stimulate and activate HSCs, induce epithelial-mesenchymal transition (EMT) of hepatic parenchymal cells, then form activated myofibroblasts and increase the synthesis of ECM such as collagen, thus promoting the occurrence of fibrosis disease (Deng et al., 2013; Lin et al., 2016; Sung et al., 2018). Therefore, therapeutic strategies targeting TGF-β signaling provide the possibility for the eventual prevention and treatment of hepatic fibrosis.

The effective treatment of hepatic fibrosis is an urgent problem to be solved. Studies have shown that combined treatment with Sorafenib and Fluvastatin can reduce collagen deposition and protein expression of α-SMA, down-regulate the content of hyaluronic acid (HA), and the expression of mesenchymal markers in rats with hepatic fibrosis induced by diethylnitrosamine (DEN). Our results suggest that combination therapy can inhibit the progression of hepatic fibrosis by inhibiting the TGF-β1/Smad3 pathway. As such, Sorafenib and fluvastatin may be a potential treatment for hepatic fibrosis. The present study found that Sorafenib can significantly inhibit the TGF-β signal, thereby inhibiting TGF-β-mediated EMT and apoptosis of hepatic parenchymal cells (Thabut et al., 2011; Ma et al., 2017). By establishing a mouse model of hepatic fibrosis induced by CCl_4, researchers found that feeding Sorafenib to model mice effectively reduced EMT and apoptosis in liver parenchymal cells, and improved and repaired hepatic fibrosis symptoms in mice (Ma et al., 2017; Sung et al., 2018). This work provides a new mechanism for Sorafenib to improve hepatic fibrosis, as well as a theoretical and experimental basis for whether the drug can be finally applied in the clinical treatment of organ fibrosis. Although Sorafenib is a potential therapeutic agent for hepatic fibrosis, it has side effects such as hand-foot syndrome, diarrhea, and hypertension due to oral administration and its non-specific uptake by normal tissues. In addition, the poor water solubility of Sorafenib decreases the efficiency of its absorption by the gastrointestinal tract, leading to poor pharmacokinetics. Future preparation formulation studies of the drug should address these issues (Lin et al., 2016).

CONCLUSION AND FUTURE DIRECTIONS

It should be noted that the development of drugs specific to hepatic fibrosis is still in its infancy. Additionally, most drugs are in phase of animal experiments or clinical trials and have various disadvantages, including lack of chronic toxicity, pharmacokinetic studies, and adequate evidence-based medicine. The occurrence and development of hepatic fibrosis is a complex process with many factors and steps. Single drugs often cause obvious adverse reactions due to large doses, single drug targets, and other factors, prices of them are often expensive. Therefore, drug combination has shown advantages of improving efficacy and reducing toxicity in clinical studies, and multi-target anti-hepatic fibrosis drugs are an important direction of future drug R&D.

Commonly used anti-hepatic fibrosis drugs include immunosuppressants, glucocorticoids, and non-specific anti-inflammatory drugs, but these drugs have more adverse reactions and poor efficacy. Moreover, due to individual differences, some patients may have adverse reactions. We found the central event in fibrogenesis appears to be the activation of HSCs, which is a complex process, leading to multiple potential targets for therapeutic interventions. Targeting only one of these targets is difficult to play an anti-hepatic fibrosis effect immediately. With the development of molecular biology, molecular targeted therapy has broad clinical application prospects beyond conventional drug therapy. Additionally, traditional Chinese medicine has unique therapeutic advantages. Therefore, the combined application of different drugs for treating hepatic fibrosis is the direction of future clinical research. Most drugs for hepatic fibrosis are still in the experimental stage. With further research on its formation mechanism and the development of new drugs, hepatic fibrosis may eventually be reversed.

AUTHOR CONTRIBUTIONS

Writing of the manuscript: LS, FW, and DZ; developing the idea for the article and critically revising it: XM and JL; supervision: XL. All of the authors have read and approved the final version of the manuscript.

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REVIEW
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Molecular Pathways and Roles for Vitamin K2-7 as a Health-Beneficial Nutraceutical: Challenges and Opportunities

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Vitamin K2-7, also known as menaquinone-7 (MK-7) is a form of vitamin K that has health-beneficial effects in osteoporosis, cardiovascular disease, inflammation, cancer, Alzheimer's disease, diabetes and peripheral neuropathy. Compared to vitamin K1 (phylloquinone), K2-7 is absorbed more readily and is more bioavailable. Clinical studies have unequivocally demonstrated the utility of vitamin K2-7 supplementation in ameliorating peripheral neuropathy, reducing bone fracture risk and improving cardiovascular health. We examine how undercarboxylated osteocalcin (ucOC) and matrix Gla protein (ucMGP) are converted to carboxylated forms (cOC and cMGP respectively) by K2-7 acting as a cofactor, thus facilitating the deposition of calcium in bones and preventing vascular calcification. K2-7 is beneficial in managing bone loss because it upregulates osteoprotegerin which is a decoy receptor for RANK ligand (RANKL) thus inhibiting bone resorption. We also review the evidence for the health-beneficial outcomes of K2-7 in diabetes, peripheral neuropathy and Alzheimer's disease. In addition, we discuss the K2-7-mediated suppression of growth in cancer cells *via* cell-cycle arrest, autophagy and apoptosis. The mechanistic basis for the disease-modulating effects of K2-7 is mediated through various signal transduction pathways such as PI3K/AKT, MAP Kinase, JAK/STAT, NF-κB, *etc*. Interestingly, K2-7 is also responsible for suppression of proinflammatory mediators such as IL-1α, IL-1β and TNF-α. We elucidate various genes modulated by K2-7 as well as the clinical pharmacometrics of vitamin K2-7 including K2-7-mediated pharmacokinetics/pharmacodynamics (PK/PD). Further, we discuss the current status of clinical trials on K2-7 that shed light on dosing strategies for maximum health benefits. Taken together, this is a synthetic review that delineates the health-beneficial effects of K2-7 in a clinical setting, highlights the molecular basis for these effects, elucidates the clinical pharmacokinetics of K2-7, and underscores the need for K2-7 supplementation in the global diet.

Keywords: vitamin K2-7, menaquinone, clinical trial, nutraceutical, osteocalcin, diabetes, neuropathy, cancer

INTRODUCTION

Chemical Structure of Vitamin K

In nature, vitamin K occurs in two main forms namely, phylloquinone (vitamin K1) and various menaquinones (vitamin K2) (Mahdinia et al., 2017). Vitamin K2 is a fat-soluble vitamin that has an ability to cross the blood brain barrier (BBB) due to its lipophilic nature (Talaei Firozjaei et al., 2018). All the existing forms of vitamin K2 consist of a common moiety in their chemical structure which is a 2-methyl-1,4-naphthoquinone ring. This core chemical structure of vitamin K2 is called menadione (**Figure 1**). Each form differs from each other with respect to their degree of saturation and length of side chains (Iwamoto, 2014). Menaquinone (also referred to as MK-n) differs in the length of its side chain varying from 4 to 15 isoprene units; n denotes the number of isoprene units present in the structure (Beulens et al., 2013). **Figure 2** depicts the chemical structures of members of the vitamin K2 family. The differences in side chains of different forms give each of them a unique potency and absorption efficiency (Booth and Rajabi, 2008). Menaquinones with absence of methyl group on naphthoquinone moiety at position 3 are called dimethyl-menaquinones and serve as precursors to menaquinones (Bentley and Meganathan, 1982).

Sources of K2-7

Main sources of phylloquinone include plant-based foods like leafy vegetables, fruits, *etc*. whereas sources of menaquinones include animal-based foods like meat, fishes, dairy products, *etc*. Short chain menaquinones like MK-4 (having four isoprene

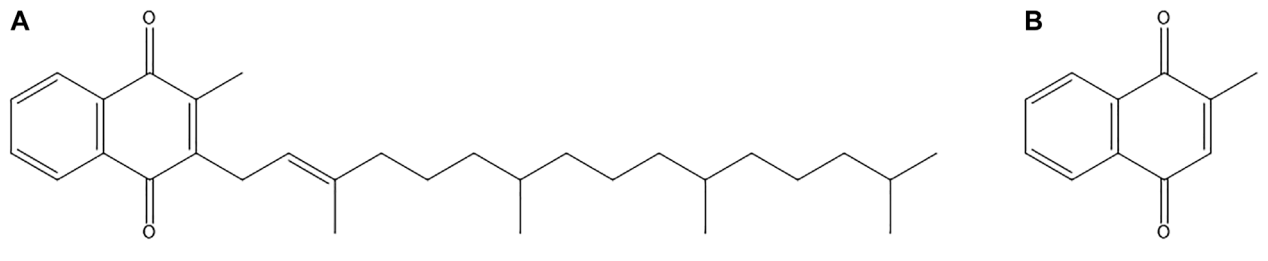

FIGURE 1 | Chemical structures of **(A)** Phylloquinone (vitamin K1); and **(B)** Menadione (vitamin K3).

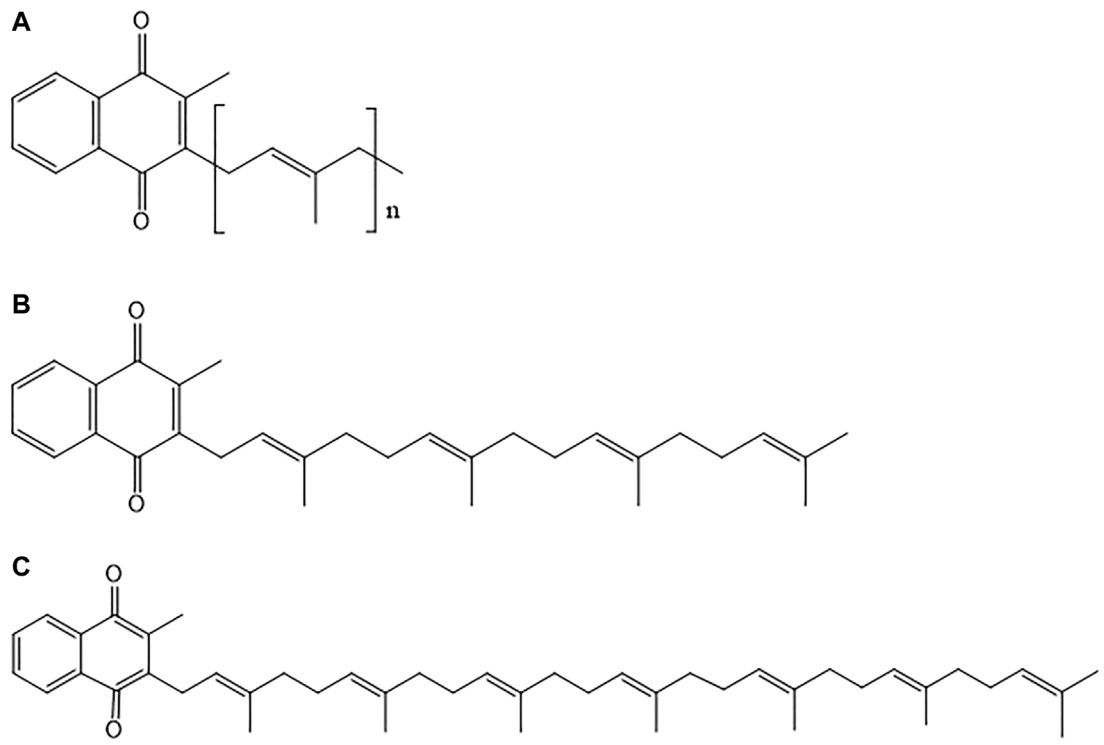

FIGURE 2 | (A) General chemical structure of vitamin K2 family; **(B)** Chemical structure of menaquinone-4 (MK-4); and **(C)** Chemical structure of menaquinone-7 (MK-7).

units) are found majorly in animal-based food products. Menaquinones having long chains such as MK-7 to MK-13 are primarily synthesized by bacteria (Shea and Booth, 2016) that include species of aerobic, anaerobic, facultative, as well as obligate anaerobic bacterial species that include *Bacillus*, *Corynebacterium*, *Escherichia*, *Lactococcus* and *Vibrio*. Some of these bacterial species are present as microflora in the gut (Bentley and Meganathan, 1982), (Conly and Stein, 1992), (Walther et al., 2013). MK-7 or K2-7 is found to be present in very high concentration in a Japanese traditional food called natto. Natto is made by fermenting soybean using *Bacillus subtilis*. Another source of menaquinones is the microflora of intestine. Although intestinal synthesis has been shown to produce significant quantities of menaquinones, absorption from this source is inefficient to fulfill the required quantities. Menaquinone is analogous to ubiquinone in function as ubiquinone also contains isoprenoid side chains of varying length, and therefore is used as an electron carrier in electron transport chain by bacteria. Depending on the dietary intake, the presence of vitamin K2-7 is also detected in human milk samples (Marles et al., 2017).

Dietary Intake of K2-7

As previously mentioned, menaquinones are originated from microbial species, and therefore, their sources majorly include dairy products and foods that are fermented exclusively by bacteria, like in natto (whole soyabeans that are fermented) (Beulens et al., 2013). The levels of K2-7 are highest in natto; moderate in chicken, sauerkraut, beef and a variety of cheeses; and low in pork, salmon, *etc* (Halder et al., 2019). Dietary intake of a population differs regionally as there is a difference of menaquinone content in food with respect to the form present in the food and the amount consumed. Mostly, availability of K2-7 from diet is low globally except in few geographic regions such as Japan and western Europe. In Japanese population, K2-7 intake is mainly due to the consumption of natto; dairy intake of western Europe population is high containing K2-7 in the fermented natural cheese, further, other forms of vitamin K2 are present in dairy products. Among all dairy products, the most important source of menaquinones is cheese. As different bacteria are used for cheese production, the content of menaquinone shows a wide variability in every cheese type (Fox et al., 2004).

Production of K2-7

Production of menaquinones is observed in bacterial cells and in animals as well. In humans, anaerobes like *Escherichia coli* present in large intestine synthesizes long chain derivatives of menaquinones (MK-7 to MK-11). Intestinally synthesized menaquinones are used as electron transporters in anaerobic respiration. Menaquinones are also produced commercially by the process of fermentation (solid state fermentation (SSF) or liquid state fermentation (LSF)) or by chemical synthesis. In SSF, the water content ranges up to 12–80%, whereas the water content is very high i.e., up to 90–95% in LSF (Mitchell et al., 2000). SSF is used for the production of secondary metabolites; the raw material used here is soy protein and corn grits. Various factors affect the production of vitamin K2-7 in SSF such as the microbial strain used, raw material or substrate along with its pre-treatment, temperature and time for which the fermentation is run. Pre-treatment of substrate is an important factor as it can increase the yield of the production. Pre-treatment using α-amylase enzyme at the first step in the fermentation process results in rise of sugar monomers and likely increases yield as well (Mahanama et al., 2011). Another important factor in the fermentation process is the selection of the bioreactor. Tray bioreactors, static deep bed bioreactors and dynamic mode fermenters that include rotating drums are some of the bioreactors used for SSF (Mahdinia et al., 2017).

Currently, liquid fermented products enjoy the favor of commercial markets. Several modifications have been researched in LSF for the fermentation media or the microbial strain or mechanical design to improve the vitamin K2-7 yield and reduce the fermentation time. Mainly *Bacillus* species including *B. subtilis* and *B. licheniformis* are studied for LSF (Mahdinia et al., 2017). Morishita *et al.* (Morishita et al., 1999) used lactic acid bacteria for the production of all vitamin K2 forms. *Leuconostoc lactis* and *Lactococcus* strains including *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* produced quinones when grown on medium containing soy milk. Therefore, these strains are used as starter cultures in dairy (Morishita et al., 1999).

Ranmadugala *et al.* studied the *Bacillus subtilis* fermentation for high production of K2-7 using biocompatible organic solvents, n-hexane and n-butanol mixture. A ~1.7-fold increase of total K2-7 was observed when compared to the control. Further, effect of 3-aminopropyltriethoxysilane-coated ferric oxide nanoparticles was studied on K2-7 production using *Bacillus subtilis* (ATCC 6633). The production as well as yield of K2-7 showed a significant increase when the strain was treated with 3-aminopropyltriethoxysilane-coated ferric oxide nanoparticles. An increase in yield by 2-fold was obtained compared to the fermentation medium containing untreated bacterial strain (Ranmadugala et al., 2018). Degree of saturation and number of isoprene units vary with the organism that synthesizes the compound (Beulens et al., 2013).

Synthesis of vitamin K2-7 results in the formation of two isomers, cis-isomer and trans-isomer. Among both the isomers, only trans-isomer has a role in biological activities. The precursor for synthesizing vitamin K2-7 is menadione, but it has harmful effects on humans. To overcome this drawback, Li *et al.* used *Pichia pastoris* strain and biologically transformed toxic menadione into vitamin K2-3 (MK-3) as an alternate precursor for the fermentation process of vitamin K2-7 (Li Z. et al., 2017). *Bacillus subtilis natto* is proven to be a promising microorganism for producing vitamin K2-7 on an industrial scale, as vitamin K2-7 produced is secreted extracellularly as well as intracellularly. Extracellularly secreted vitamin K2-7 is bound with vitamin K2-binding factor, a protein complex, which is soluble in the fermentation broth. Biosynthesis pathway initiates with shikimic acid and ends at vitamin K2-7 involving seven intermediates. Fermentation in static culture results in formation of biofilm and growth in the form of pellicle (Mahdinia et al., 2019).

Mahdinia *et al.* studied the key growth factors required for improving the production of vitamin K2-7 in biofilm reactors using *B. subtilis natto*. The growth factors included optimum pH,

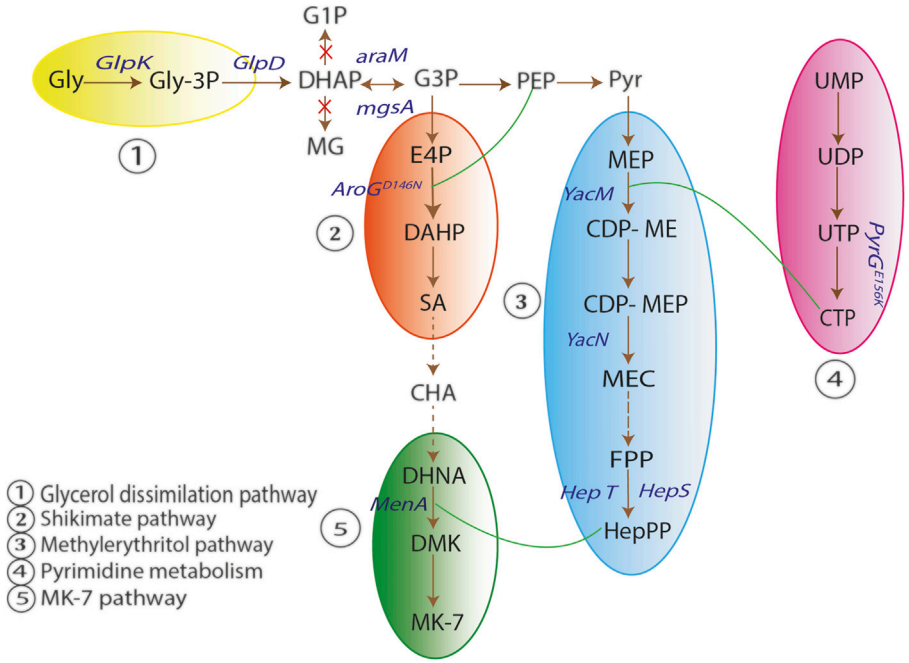

FIGURE 3 | Schematic representation of vitamin K2-7 synthesis. In glycerol dissimilation pathway, overexpression of enzymes glycerol kinase (GlpK) and glycerol-3-phosphate dehydrogenase (GlpD) leads to conversion of glycerol (Gly) to dihydroxyacetone phosphate (DHAP). Conversion of DHAP to methylglyoxal (MG) and glyceraldehyde-1-phosphate (G1P) is prevented by deleting mgsA and araM. DHAP is interconverted to glyceraldehyde-3-phosphate (G3P) which is converted to pyruvate (Pyr). Pyr enters the methylerythritol pathway (MEP) in which the end product is heptaprenyl pyrophosphate (HepPP). Synthesis of HepPP is enhanced by overexpression of enzyme heptaprenyl diphosphate synthase (HepS). HepPP enters MK-7 pathway to form MK-7 (vitamin K2-7). $AroG^{D146N}$ and $PyrG^{E156K}$ are overexpressed in shikimate (SA) pathway and pyrimidine metabolism pathway respectively to lead the components of both the pathways towards MK-7 pathway and form MK-7 as the end product (Adapted from (Yang et al., 2020)).

temperature and agitation. The medium used for the production was glycerol-based as it is relatively cheaper compared to other carbon sources like sucrose, glucose and mannose and can also improve the production of vitamin K2-7. The optimum growth parameters reported were pH: 6.58, temperature: 35°C and agitation: 200 rotations per minute (rpm). An increase of 58% in the concentration of vitamin K2-7 was observed when growth parameters were optimized (Mahdinia et al., 2018).

Yang *et al.* constructed a metabolically engineered *B. subtilis* strain for enhancing the production of MK-7. In *B. subtilis*, synthesis of vitamin K2-7 is divided into five pathways, *viz.*, glycerol dissimilation/dissociation pathway, shikimic acid (SA) pathway, methylerythritol phosphate (MEP) pathway, pyrimidine metabolism pathway and vitamin K2-7 (MK-7) pathway. In glycerol dissimilation pathway, overexpression of enzymes like glycerol kinase (GlpK) and glycerol-3-phosphate dehydrogenase (GlpD) led to conversion of glycerol to dihydroxyacetone phosphate (DHAP). Further, conversion of DHAP into methylglyoxal (MG) and glyceraldehyde-1-phosphate (G1P) was prevented by deleting *mgsA* and *araM*. DHAP is interconverted to glyceraldehyde-3-phosphate (G3P) which further converts into pyruvate and enters MEP pathway wherein the end product is heptaprenyl pyrophosphate (HepPP). Synthesis of HepPP was enhanced by overexpressing enzyme heptaprenyl diphosphate synthase (HepS). HepPP then entered MK-7 pathway to form vitamin K2-7. In addition, $AroG^{D146N}$ and $PyrG^{E156K}$ were overexpressed in SA pathway and pyrimidine metabolism pathway respectively to lead the components of both the pathways towards MK-7 pathway and ultimately form MK-7 as the end product in high concentrations as shown in **Figure 3** (Yang et al., 2020).

Gao *et al.* reported the use of genetically and metabolically engineered *Escherichia coli* for production of vitamin K2-7. Under aerobic conditions, ubiquinone-8 (Q-8) is produced by *E. coli*, and, under anaerobic conditions, it produces vitamin K2-8 instead of vitamin K2-7, because of the presence of octaprenyl diphosphate synthase which is encoded by *IspB*. For synthesizing one molecule of vitamin K2-7, seven molecules of isopentenyl diphosphate (IPP), the building blocks of isoprenoid units, are required. In mevalonic acid pathway, acetyl-CoA is converted to IPP which is further converted into vitamin K2-7 *via* heptaprenyl pyrophosphate. This study was carried out to produce engineered *E. coli* by introducing heptaprenyl pyrophosphatase synthetase (HepPPS) enzyme derived from *B. subtilis*. Further, production of vitamin K2-7 was enhanced by overexpressing HepPPS and optimizing the expression of enzymes in mevalonic acid pathway in order to eliminate synthesis of metabolites other than those involved in K2-7 pathway (Gao et al., 2020). In another study Gao *et al.* investigated an alternative procedure for highly efficient production of vitamin K2-7 by using

TABLE 1 | Synthetic pathways in production of vitamin K2-7.

Synthetic pathway	Advantages	Disadvantages	References
A. Genetic and metabolic engineering			
*1. E. coli (*pLB1s-ESK1)	High yield of MK-7; has a short production cycle	Requires optimization	Gao et al. (2020)
2. B. subtilis	Best MK-7-producing recombinant microbe	High cost of *B. subtilis* fermentation; has a long production cycle	(Ma et al., 2019; Gao et al., 2020)
B. Fermentation			
1. Soy protein granules and nixtamalized corn grits	Higher yield of MK-7 and low manufacturing costs	—	Mahanama et al. (2011)
2. Lactococcus lactis ssp. cremoris, Lactococcus lactis ssp. lactis, Leuconostoc lactis	Higher yield of MK-7	—	Morishita et al. (1999)
3. B. subtilis natto	Improved production of MK-7	Formation of pellicles which can be problematic and undesirable during fermentation	(Berenjian et al., 2013)

TABLE 2 | Genes modulated by vitamin K2-7 in various diseases.

Conditions	Genes	Expression	References
Osteoporosis	*biglycan*	Downregulated	Katsuyama et al. (2007)
	butyrophilin	Downregulated	Katsuyama et al. (2007)
	tenascin C	Upregulated	Katsuyama et al. (2007)
	BMP2	Upregulated	Katsuyama et al. (2007)
Vascular calcification	*BMP2*	Downregulated	Boström et al. (1993)
Cancer	*cyclin D1*	Downregulated	Xia et al. (2012)
Diabetes	*TNFα*	Downregulated	Li et al. (2018)
	IL-1β	Downregulated	Li et al. (2018)
	IL-6	Downregulated	Li et al. (2018)
Peripheral neuropathy	*TNFα*	Downregulated	Pan et al. (2016)
	IL-1β	Downregulated	Pan et al. (2016)
Alzheimer's disease	*TNFα*	Downregulated	Saputra et al. (2019)
	IL-1β	Downregulated	Saputra et al. (2019)
	IL-6	Downregulated	Saputra et al. (2019)

metabolically engineered *E. coli* under aerobic conditions (Gao et al., 2021).

We have enlisted the synthetic pathways involved in production of vitamin K2-7 in **Table 1** along with their advantages and disadvantages.

VITAMIN K2-7 AND ASSOCIATED DISEASES

Vitamin K2-7 plays an important role in various biological functions along with vitamin K-dependent proteins (VKDPs). K2-7 is a cofactor of enzyme γ-carboxylase which drives the conversion of inactive VKDPs (such as osteocalcin and matrix Gla protein) to their active forms. Vitamin K2 helps in carboxylation of glutamate (Glu) residues present on VKDPs to γ-carboxyglutamate (Gla) which results in their activation. In the absence of vitamin K2, the optimal functioning of these proteins is hindered which leads to pathological complications that include metabolic conditions like diabetes and chronic degenerative conditions (cardiovascular disease, osteoporosis, *etc*) (Vaidya et al., 2022b). Genes modulated by vitamin K2-7 in various diseases are summarized in **Table 2**.

Vitamin K2-7 and Osteoporosis

Osteoporosis is the most common bone disorder found in the older population (Mandatori et al., 2021). An imbalance between bone formation and bone resorption causes this metabolic disorder leading to depletion of bone mass, deterioration of skeletal structure and an elevated risk of bone fractures (Hendrickx et al., 2015). Bone homeostasis is maintained by osteocytes, osteoblasts and osteoclasts. Bone formation is promoted by osteoblasts and bone resorption is stimulated by osteoclasts. Decreased formation and increased resorption of bone results in bone loss with aging. Osteoporosis is caused by bone loss along with various pathophysiologic states. K2-7 aids in stimulation of osteoblastic formation of bone and suppression of osteoclastic resorption of bone. In osteoblastic cells, K2-7 helps in protein synthesis of osteocalcin and various other proteins. Interestingly, the ratio of circulating K2-7 serum levels between eastern Japanese women and British women is 15:1 which inversely mirrors the fracture rate of 1 in Japanese women to that of 15 in British women (Kaneki et al., 2001). This implies a strong beneficial effect of K2-7 circulating levels in reducing the risk of fractures globally.

Cellular functions in osteoclastic and osteoblastic cells are performed by various proteins, whose expression is regulated by K2-7 (Yamaguchi, 2014). Osteocalcin produced by osteoblasts binds to calcium present in blood circulation and leads it to the bone matrix. Bone mineralization is influenced by osteocalcin as it has high affinity towards hydroxyapatite, a mineral component of bone; this results in stronger skeleton and less susceptibility to fracture (Hoang et al., 2003). The newly synthesized osteocalcin is inactive and it requires vitamin K2-7 for converting itself into active form by carboxylation, and later bind to calcium (Hauschka et al., 1989). Vitamin K2-7 is a cofactor of enzyme γ-carboxylase, that converts glutamic acid (Glu) residues present in the molecule of osteocalcin (OC) to γ-carboxyglutamate (Gla) and is, therefore, necessary for the γ-carboxylation of OC. Thus,

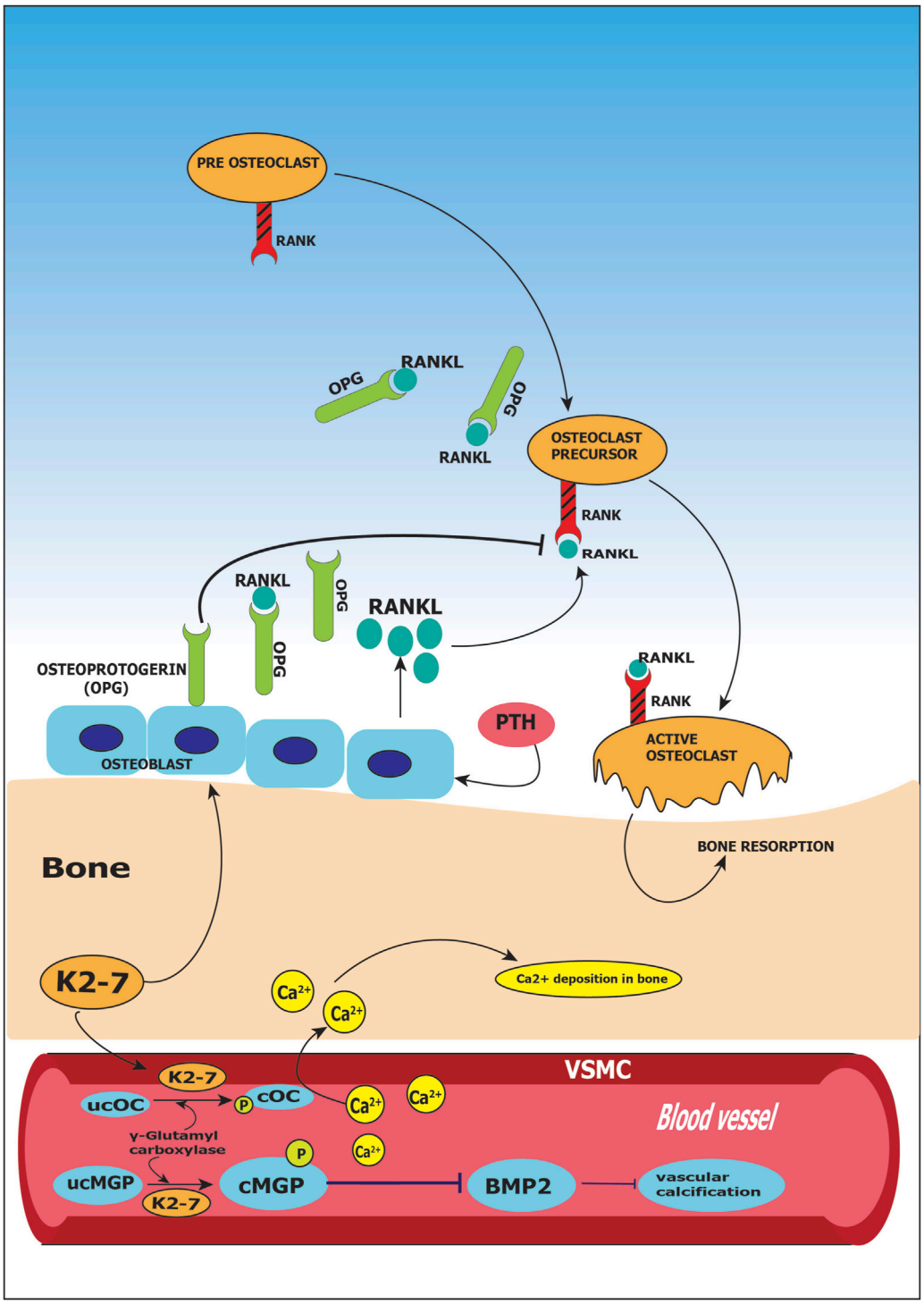

FIGURE 4 | Role of vitamin K2-7 in osteoporosis and vascular calcification. Vitamin K2-7 facilitates carboxylation of glutamate (Glu) residue present on matrix Gla protein (MGP) leading to formation of carboxylated MGP (cMGP) and aids in phosphorylation of MGP leading to its activation; activated MGP inhibits bone morphogenetic protein 2 (BMP2) resulting in prevention of vascular calcification. K2-7 also is a cofactor in conversion of undercarboxylated osteocalcin (ucOC) to carboxylated osteocalcin (cOC); cOC has an affinity for calcium ions and facilitates the transport of calcium to bone for bone formation. Receptor activator for nuclear factor kappa ligand (RANKL) binds to RANK receptor and activates osteoclasts which results in bone resorption; vitamin K2-7 augments the expression of osteoprotegerin (OPG) which is a decoy receptor for RANKL and abrogates RANK-RANKL binding thus inhibiting bone resorption.

vitamin K2-7 is thought to be involved in bone mineralization as an essential element for the γ-carboxylation of OC (Iwamoto et al., 2006). K2-7 by upregulating osteoprotegerin, a decoy receptor for RANKL, prevents bone resorption. RANKL otherwise binds to receptor activator of NF-κB (RANK) and activates nuclear factor kappa beta (NF-κB). Activation of NF-κB is essential for osteoclasts proliferation which results in osteoporosis through bone resorption. Thus, RANK receptor is sequestered by K2-7 preventing steps leading to osteoporosis shown in **Figure 4** (Badmaev et al., 2011).

Wu *et al.* performed a comparative study of vitamin K2-7 effect, isolated from *cheonggukjang*, with vitamin K2-4 and vitamin K1. The effect of all three vitamins was determined on mineralization and cell differentiation of the pre-osteoblastic cell line MC3T3-E1 obtained from neonatal mouse calvariae. Vitamin K2-7, vitamin K2-4 and vitamin K1 significantly enhanced activity of alkaline phosphatase (ALP) that resulted in bone formation; increased proliferation of osteoblastic cell line MC3T3-E1 was also reported. It was observed that vitamin K2-4 and K2-7 had comparatively more dynamic effect than vitamin K1; this suggested that vitamin K2 (K2-4 and K2-7) and vitamin K1 probably have different mechanisms for stimulation of mineralization of osteoblasts. Furthermore, the mRNA expression ratio of osteoprotegerin and RANKL was also upregulated after treatment with 10 μM of vitamin K2 (K2-4 and K2-7) for 24 h. This indicates that vitamin K has a potential role in suppression of osteoclast formation by upregulating mRNA expression ratio of osteoprotegerin and RANKL (Wu et al., 2019).

Katsuyama *et al.* (Katsuyama et al., 2007) using MC3T3E1 osteoblastic cells obtained from calvaria of newborn mouse studied the differential expression of genes after treatment with vitamin K2-7. Both control and vitamin K2-7-treated MC3T3E1 cells were analyzed. Downregulation of *biglycan* and *butyrophilin* genes and upregulation of *tenascin C* and *BMP2* were observed after 24 h of treatment with K2-7. Tenascin C is an important protein involved in bone remodeling as it promotes differentiation of osteoblastic cells. BMP2 signaling is responsible for the production of osteoblast-specific proteins such as osteocalcin. Indeed, MK-7 induces the production of osteoprotegerin, osteocalcin receptor activator of NFκB (RANK), and its ligand (RANKL) in osteoblastic MC3T3E1 cells, thus indicating that vitamin K2 may play an important role in bone homeostasis. Biglycan is a member of the class I family of small leucine rich proteoglycans (SLRPs). Studies have established the role of biglycan in osteoblast differentiation and matrix mineralization through the BMP4 signaling pathway (Nastase et al., 2012). *In vitro* studies have shown that biglycan interacts with other BMPs, such as BMP2 and 6. Biglycan is able to directly bind BMP2 and its receptor, ALK6 (also known as BMP-RIB), to stimulate BMP2- dependent osteoblast differentiation (Moreno et al., 2005). Butyrophilin is associated in regulating B7 protein (Malinowska et al., 2017) which is involved in osteoblast differentiation (Suh et al., 2004, 3).). It was observed that vitamin K2-7 increased tenascin C levels through BMP2 pathway which has a role in autocrine signaling. Tenascin C levels increased by ~1.5 fold when treated with 10^{-5} M vitamin K2-7 and ~3 fold when treated with 10^{-6} M of vitamin K2-7. In addition, increase in phosphorylated Smad1 level was reported in cells treated with 10^{-6} M vitamin K2-7. As nuclear binding receptor for vitamin K2-7 is present on MC3T3E1 cells, it is possible that, through production of BMP2, K2-7 has an indirect effect on the BMP2-Smad1 pathway.

Gigante *et al.* (Gigante et al., 2015) studied the effect of vitamin K2-7 (with and without vitamin D3) on differentiation of human mesenchymal stem cells (hMSCs) obtained from bone marrow. Vitamin K2-7 enhances the process of gene induction of osteocalcin that is initially influenced by vitamin D3. It was observed that vitamin K2-7 has an effect on genes involved in cell growth and cell differentiation which included growth differentiation factor-10 (*GDF10*) and insulin-like growth factor 1 (*IGF1*). In addition, co-supplementation of vitamin K2-7 influenced vascular endothelial growth factors (VEGFA) induction along with its receptor fms-related tyrosine kinase 1 (FLT1); FLT1 plays a role in both angiogenic and osteogenic processes. Co-supplementation of vitamins aided in bone-healing process by modulating the expression of genes involved in both mineralization and angiogenesis. Considering genes involved in bone formation and mineralization, vitamin K2-7 enhanced vitamin D3 gene induction of osteocalcin. Hence, co-supplementation strategy has the potential to help in better development of bone and reduce bone-related disorders.

Vitamin K2-7 and Vascular Calcification

Vascular calcification is characterized by mineral depositions on the walls in the vascular system; the depositions present are of calcium phosphate complexes in the form of hydroxyapatite (El Asmar et al., 2014). Based on recent clinical and pre-clinical studies, Abdullah et al. highlighted the potential cellular and physiological roles of vitamin K in cardiovascular diseases (CVD), and highlighted the association between CVD prevention and vitamin K supplementation (Al-Suhaimi and Al-Jafary, 2020). Matrix Gla protein (MGP) plays a key role in inhibition of vascular calcification; it has the potential to inhibit as well as to reverse the process of calcification. MGP undergoes post-translational modifications like phosphorylation and carboxylation which result in the activation of MGP from its inactive state. Serine residues present on MGP are phosphorylated by casein kinase, this results in secretion of MGP in the extracellular matrix (Roumeliotis et al., 2019). In addition, activation of MGP takes place when glutamate residues present on MGP undergo carboxylation by γ-carboxylase. Vitamin K2-7 is the cofactor of γ-carboxylase, hence, plays an important role in activation of MGP. Negatively charged MGP has high affinity towards free calcium present in the blood vessels. It binds directly to the circulating calcium and hydroxyapatite crystals that are accumulated in the walls of the vessels forming inactive complexes (Roumeliotis et al., 2020). Negatively charged MGP has high affinity towards free calcium present in the blood vessels. It binds directly to the circulating calcium and hydroxyapatite crystals that are accumulated in the walls of the vessels forming inactive complexes (Roumeliotis et al., 2020). MGP initiates autophagic clearance by attracting

macrophages and phagocytes (Shanahan, 2005). In addition, MGP removes free circulating calcium and leads it to the bone. Furthermore, MGP inhibits vascular calcification through downregulation of bone morphogenetic protein-2 (BMP-2) which promotes vascular calcification. Transformation of VSMCs to an osteoblastic phenotype is triggered by BMP-2 and is found within the walls of calcified arteries (Boström et al., 1993). MGP is in inactive state and requires vitamin K2-7 for γ-carboxylation of its glutamic acid (Glu) into γ-carboxyglutamate (Gla). When Glu is transformed into Gla, molecular changes occur in the structure of MGP which in turn activates it. Further, to become biologically active, MGP undergoes phosphorylation of serine residues. Phosphorylation is dependent on vitamin K and is considered as the most critical step in activation of MGP. Therefore, to gain the ability to bind to hydroxyapatite, calcium and BMP-2 MGP is required to be carboxylated as well as phosphorylated (**Figure 4**) (Wallin et al., 2000). Furthermore, vascular calcification is also associated with activation of growth arrest-specific gene 6 (Gas6) activated by vitamin K2. Gas6 undergoes γ-carboxylation by vitamin K2 to trigger anti-apoptotic activity of Bcl-2. Gas6 also inhibits caspase 3, a pro-apoptotic protein, thus preventing the apoptosis induced by calcification and starvation of fibroblasts (Villa et al., 2017). Gas6 and other growth factors act as growth promoters in VMSCs.

Vascular calcification is a significant predictor of cardiovascular disease. As mentioned earlier, MGP plays a crucial role in inhibition of vascular calcification. Through γ-carboxylation, vitamin K2 may help reduce vascular calcification and thereby reduce the risk of cardiovascular disease (Beulens et al., 2013). Patients suffering from chronic kidney disease often build up complications like vascular calcification and bone disorders that are caused by mineral disturbances (Schlieper et al., 2016). Thus, phosphate retention occurs as the function of kidney becomes less efficient. For the transformation of VMSCs into osteoblast-like cells, phosphate plays a role as key signaling mediator. Arterial wall mineralization is mediated by bone matrix proteins that are produced by osteoblast-like cells (Leopold, 2015). Vitamin K2 carboxylates MGP to prevent vascular calcification and in turn abrogates complications in chronic kidney disease.

Vitamin K2-7 and Cancer

Several therapeutics are available for patients suffering from cancer, yet prognosis for the long term is inadequate for different types of cancer. Vitamin K2 exerts anti-cancer effects on various cell lines such as leukemia, hepatocellular carcinoma (HCC), lung cancer, ovarian cancer, pancreatic cancer and colorectal cancer (Xv et al., 2018). Cancerous cell growth is suppressed by vitamin K2 *via* apoptosis, autophagy and cell-cycle arrest.

Vitamin K2 has the ability to inhibit proliferation of cancer cells through induction of cell-cycle arrest, here the activity of NF-κB is inhibited which plays an important role. NF-κB is a nuclear transcription factor and associates itself with cell growth by regulation of the *cyclin D1* gene (Xia et al., 2012). During cell cycle, Cyclin D1 contributes to the G_1-S transformation by binding to CDK4 or CDK6 (Masaki et al., 2003). The expression of cyclin D1 is downregulated by vitamin K2 through preventing NF-κB from binding to cyclin D1 promoter (Xia et al., 2012). Another mechanism by which vitamin K2 shows its anticancer effect is by inducing apoptosis of cancer cells. Mitochondrial apoptosis is induced by vitamin K2 *via* mitogen-activated protein kinase (MAPK) pathways (Tsujioka et al., 2006; Kanamori et al., 2007; Sibayama-Imazu et al., 2008; Showalter et al., 2010). Extracellular signal-related kinases (ERKs) stimulate cell proliferation and inhibit cell-death signals in response to mitogenic signals or growth factors whereas, c-Jun N-terminal kinase (JNK) pathway and p38 MAPK pathway are implicated in apoptosis and inflammation. Activation of p38 is carried out by vitamin K2 by phosphorylating p38 (Olson and Hallahan, 2004; Tsujioka et al., 2006). Vitamin K2 can inhibit cancer cell growth by inducing autophagy. Vitamin K2 has the ability to simultaneously cause autophagy and apoptosis in leukemic cells; when high expressions of Bcl-2 are observed, autophagy is comparatively more dominant, this restrains apoptosis. Therefore, autophagy can be an alternative for inducing apoptosis instead of distinct form of cell death (Yokoyama et al., 2008).

In the cell cycle, proto-oncogenic protein Cyclin D1 regulates the G_1 to S phase transition by phosphorylation of pRb and binding to Cdk4 or Cdk6 (Hall and Peters, 1996). Synthesis of DNA and contact-independent growth in several human cancers including hepatocellular carcinoma (HCC) can be enhanced when cyclin D1 is overexpressed (Zhang et al., 1993; Hall and Peters, 1996; Masaki et al., 2003). Expression of *Cyclin D1* gene is regulated by NF-κB along with several other factors (Guttridge et al., 1999; Joyce et al., 2001). Ozaki *et al.* (Ozaki et al., 2007) investigated the effect of vitamin K2 on growth inhibition of HCC cells (Huh7, HepG2 and Hep3B). Expressions of growth-related genes that included cyclin D1 and cyclin-dependent kinase inhibitors were evaluated at the protein and mRNA levels after HCC cells were treated with different concentrations of vitamin K2 ranging from 10^{-4} to 10^{-7} M for 48 h. G_1 phase arrest was observed in HCC cells treated with vitamin K2. Cyclin D1 protein and mRNA expression was downregulated by vitamin K2 treatment. Moreover, the expressions of Cdk inhibitors (p21 and p27) were elevated in HepG2 cells. This indicated that during vitamin K2-induced growth inhibition in HCC cells, the expressions of cyclin D1 and Cdk inhibitors is regulated. Activity of cyclin D1 promoter was examined to further study the effects of vitamin K2. Vitamin K2 inhibited the cyclin D1 promoter activity significantly in all the three cell lines in a dose-dependent manner. Inhibition of activity of cyclin D1 promoter by vitamin K2 is dependent on NF-κB. Vitamin K2 also contributed to the inhibition of both basal and induced NF-κB binding activities by inhibiting the activity of inhibitory κB kinase (IKKα). Hence, vitamin K2 inhibited HCC cells growth by suppressing expression of cyclin D1 *via* IKK/NF-κB pathway and, therefore, can prove to be useful for treating HCC.

The post-translational carboxylation of the prothrombin precursor is dependent on vitamin K-dependent γ-glutamyl carboxylase. HCC cells are unable to carboxylate all Glu residues due to carboxylase deficiency (Uehara et al., 1999).

Prothrombin Induced by Vitamin K absence-II (PIVKA-II), also known as des-gamma-carboxy prothrombin (DCP), is an abnormal prothrombin precursor produced in HCC. It is a potential autologous growth stimulator for HCC proliferation and has insufficient coagulant activity. PIVKA-II is considered as a potential serum biomarker for HCC with better diagnostic performance and higher specificity in early detection of HCC (Feng et al., 2021). PIVKA-II in combination with serum α-fetoprotein (AFP) enhanced the accuracy of surveillance of HCC in high-risk populations. Additionally, PIVKA-II acts as a marker for the assessment of response to treatment in HCC (Yang et al., 2021). Hitomi et al. investigated the antitumor effects of vitamin K2 on athymic BALB/c-nu/nu mice. Vitamin K2 (400 μM) was administered orally for 53 days from the day after inoculation of PLC/PRF/5 human HCC cells. It was observed that there was significant decrease in tumor size (five fold). In addition, there was significant reduction in the protein expression of cyclin D1 and Cdk4 in HCC suggesting G1 arrest of the cell cycle. However, Cdk inhibitor $p16^{INK4a}$ was not affected by vitamin K2 (Hitomi et al., 2005; Al-Suhaimi, 2014). Furthermore, Lu et al. studied the inhibition of HCC cell proliferation using vitamin K2 in HepG2 cells. 17β-hydroxysteroid dehydrogenase 4 (HSD17β4) is a protein that promotes cell proliferation. When HSD17β4 is overexpressed, it promotes the cell proliferation of HCC. Vitamin K2 binds directly to the protein, without affecting its gene expression, and inhibits MEK/ERK and Akt signaling pathways which further result in inhibition of HCC cell proliferation (Lu et al., 2021).

Vitamin K2-7 and Diabetes

Diabetes mellitus type 2 (T2DM) which is associated with macro- and micro-vascular complications is a major public health problem around the world. We have recently delineated the noncoding RNA interactome including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) in the pathogenesis of diabetes (Pandey et al., 2022). In T2DM, insulin sensitivity is improved by vitamin K2 through involvement of osteocalcin (vitamin K-dependent protein), anti-inflammatory properties, and lipid-lowering effects (Zhang et al., 2017; Li et al., 2018). Vitamin K2 improves sensitivity to insulin in diabetic patients *via* metabolism of osteocalcin which has a role in increasing adiponectin expression. In addition, administration of vitamin K2 results in alteration of NF-κB and GSK-3β expression involved in chronic inflammation and adiponectin regulation respectively (Otani et al., 2015; Zhang et al., 2017). Undercarboxylated osteocalcin (ucOC) is an active hormone that affects glucose metabolism. It is an important biomarker for vitamin K status and is associated with diabetes (Lin et al., 2018). Supplementation of vitamin K2 increased carboxylation of osteocalcin (Berkner, 2005), and it was postulated that osteocalcin influenced insulin sensitivity *via* adiponectin regulation. Osteocalcin signaling pathway regulated glucose metabolism which led to an increase in insulin1 (Ins1) and insulin2 (Ins2) gene expression (Hussein et al., 2018; Al-Suhaimi and Al-Jafary, 2020). In the liver, phosphorylation of peroxisome proliferator-activated receptors (PPAR-α), p38 MAP kinase and 5′AMP-activated protein kinase (AMPK) is increased as adiponectin receptor 2 mediates the effect of adiponectin. In addition, the effect of globular adiponectin is controlled by adiponectin receptor 1 resulting in increased phosphorylation of PPAR-α, p38 MAPK and AMPK. It was observed that insulin sensitivity was also augmented with increase in oxidation of fatty acids and glucose uptake (Weyer et al., 2001; Yamauchi et al., 2003; Lihn et al., 2005). Response to inflammation has a role in etiology as well as pathogenesis of T2DM. IL-6, a proinflammatory mediator, plays a role in activation of suppressor of cytokine signaling (SOCS) protein, this in turn blocks the activation of insulin transcription factor. Signal transducer and activator of transcription 5B (STAT5B) protein binds to insulin receptor and activates insulin transcription factor. SOCS competes with STAT5B that blocks the activation of insulin transcription factor (Emanuelli et al., 2000). Vitamin K2 inhibits inflammatory responses *via* NF-κB signaling pathway inactivation, which plays a key role in suppressing the expression of IL-6, IL-1β, TNF-α. Thus, by suppressing inflammatory responses, vitamin K2 improves insulin resistance (Li et al., 2018).

Vitamin K2-7 and Peripheral Neuropathy

Peripheral neuropathy is a disorder characterized by numbness, slow nerve conduction, pain and tingling sensations in the limbs (Head, 2006). One of the causes of peripheral neuropathy is demyelination of peripheral nerve fibres. Demyelination results in deterioration of the structural and molecular features of the nerve fibres, which leads to peripheral neuropathy (Wei et al., 2019). We have recently reviewed the intrinsic need for vitamin K2-7 supplementation in peripheral neuropathy (Vaidya et al., 2022a). We have also recently elucidated the miRNA regulatory interactome in neuropathic pain (Gada et al., 2022). Vitamin K2 facilitates synthesis and repair of the myelin sheath in the peripheral nervous system. In addition, vitamin K2 facilitates the activation of Gas6 by carboxylating the Gla residues of Gas6, which is structurally related to anticoagulation factor protein S. Gas6 and protein S bind to form a complex and activate the receptor tyrosine kinase of TAM (Tyro3, Axl, and Mer) family which leads to increased myelin production and repair after myelin injury (Mehta, 2017). Proinflammatory cytokines like TNFα and IL-1β are associated with peripheral neuropathy development through neuroinflammatory mechanisms (Li QY. et al., 2017). Vitamin K2 is able to inhibit gene expression of TNFα and IL-1β in human monocyte-derived macrophages in a dose-dependent manner (Pan et al., 2016). In bones, primary activation of the RANKL/RANK (receptor activator for nuclear factor kappa B) system activates osteoclasts, which triggers the damage of bones and subsequently cause damage to the peripheral sensory nerves around bones due to bone fracture. Neuropathic pain develops when there is a damage in peripheral nerves (Zajączkowska et al., 2019). RANKL/RANK system activates NF-κB, a regulator of inflammation, leading to osteoclasts activation. Vitamin K2-7 prevents activation of the RANKL/RANK system by upregulating osteoprotegerin, a decoy receptor for RANKL. Thus, by preventing the binding of RANKL to the RANK receptor, vitamin K2 abrogates activation of NF-κB and hence activation of osteoclasts (Badmaev et al., 2011).

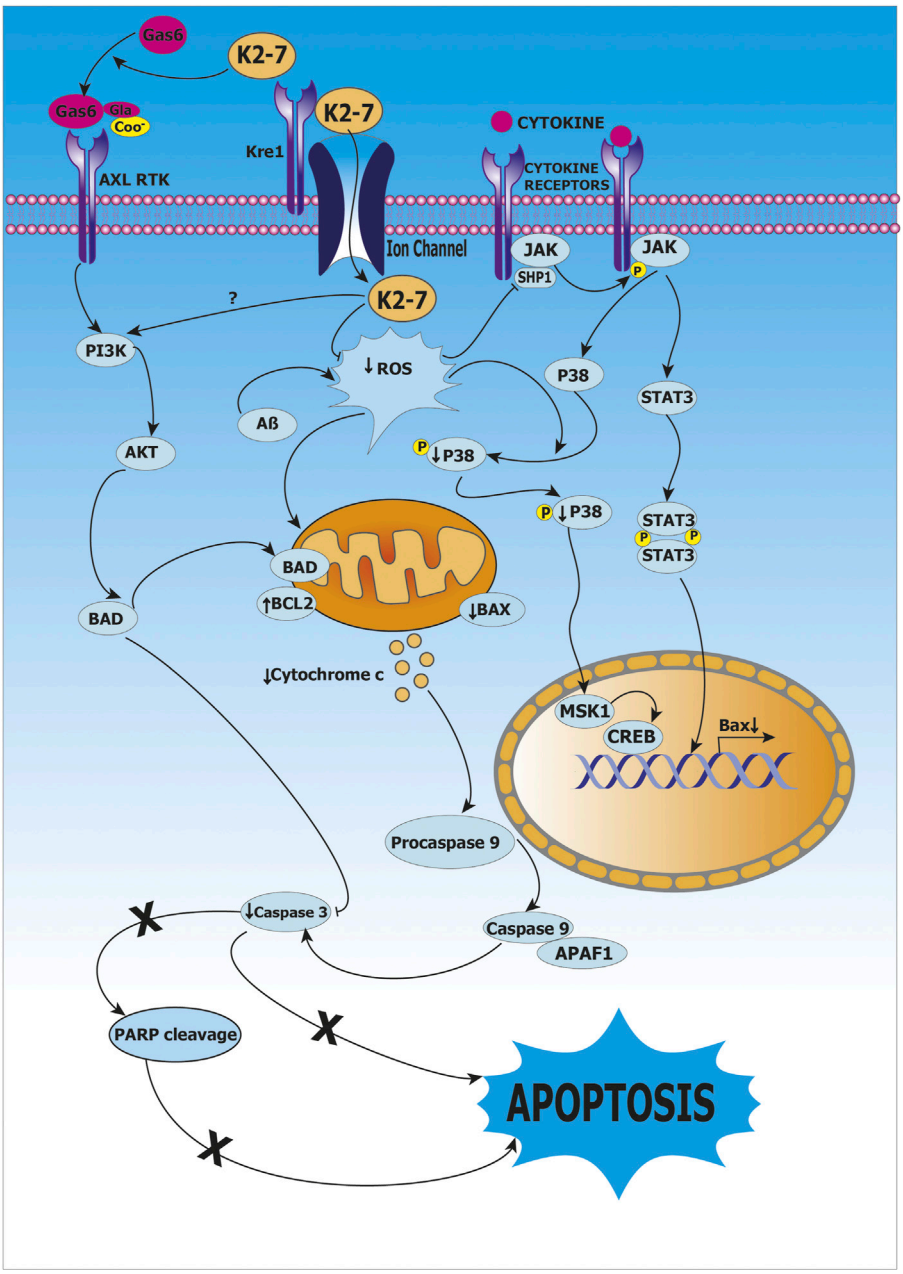

FIGURE 5 | Role of vitamin K2-7 in Alzheimer's disease. Vitamin K2-7 facilitates carboxylation of glutamate (Glu) residue present on growth arrest-specific protein 6 (Gas6) to γ-carboxyglutamate (Gla) leading to its activation, activated Gas6 binds to AXL receptor tyrosine kinase (AXL RTK) and initiates phosphoinositide 3-kinase/protein kinase B/BCL-2-associated death promoter protein (PI3K/AKT/BAD) signaling, BAD has an inhibitory effect on Caspase 3, this abrogates apoptosis and provides protection by K2-7 against β-amyloid (Aβ)-induced cytotoxicity. In cytosol, reactive oxygen species (ROS) activates Janus kinase-Signal transducer and activator of transcription protein (JAK-STAT) pathway resulting in transcription of BCL-2-associated X protein (BAX), BAX causes release of cytochrome c from mitochondria, cytochrome c leads to activation of Caspase 3 that results in apoptosis; vitamin K2-7 abrogates apoptosis by inhibiting Caspase 3.

Considering these properties of vitamin K2-7, it may likely serve as an effective intervention in the management of neuropathic pain.

Vitamin K2-7 and Alzheimer's Disease

According to Alzheimer's Foundation of America, "Alzheimer's disease is a progressive brain disorder that impacts memory, thinking and language skills, and the ability to carry out the simplest tasks." (Alzheimer's Foundation of America). The levels of vitamin K2 in serum level were reduced in patients suffering from AD (Huy et al., 2013). Vitamin K2 has the potential to slow down the progression of AD and contribute to its prevention.

Hadipour *et al.* (Hadipour et al., 2020) studied the effects of vitamin K2 at concentrations ranging from 5 to 200 μM in rat

pheochromocytoma PC-12 cells to provide protection against toxicity caused by hydrogen peroxide and β-amyloid. Vitamin K2 reduced the cytotoxicity caused by hydrogen peroxide and β-amyloid. In Alzheimer's disease, β-amyloid led to neuronal death by direct toxicity and by promotion of apoptosis; whereas vitamin K2 prevented neuronal death resulting from β-amyloid in PC-12 cells. The cells that were pre-treated with vitamin K2 exhibited remarkably less apoptosis when the cells were exposed to either hydrogen peroxide or β-amyloid. Further, vitamin K2 also decreased the levels of reactive oxygen species (ROS) in PC-12 cells that were exposed to hydrogen peroxide and β-amyloid. The levels of glutathione, an antioxidant, also increased when cells were pre-treated with 20 and 50 μM of vitamin K2. Hence, vitamin K2 pre-treatment reduced apoptosis signaling proteins (β-amyloid, caspase 3, *etc.*), attenuated levels of ROS, and increased levels of glutathione. In addition, it was reported that PC-12 cells when treated with hydrogen peroxide and β-amyloid increased phospho-p38 MAP kinase, PARP cleavage and Bax/Bcl-2 ratio; while pre-treatment with vitamin K2 resulted in decreased phosphorylation of p38 MAPK, PARP cleavage and mRNA level of Bax/Bcl-2. Inactivation of p38 MAPK pathway was identified as a mechanism for potential protective role of vitamin K2 in AD. Hence, the study confirms the protective role of vitamin K2 mediated by its antioxidant and anti-apoptotic properties (Hadipour et al., 2020).

Huang *et al.* studied the role of vitamin K2 as a protective agent using clones of rat astroglioma C6 cells that were transfected with C-terminal fragment of β-amyloid precursor protein (β-CTF/C6 cell model). Treatment of β-CTF/C6 cells with 0–10 μM vitamin K2 and 250 μg cumate for 72 h resulted in increased phosphorylation of Bcl-2-associated death promoter protein (Bad) and decrease in apoptosis mediated by caspase 3, this indicated that caspase 3-mediated apoptosis was inhibited by vitamin K2 *via* activation of protein Bad (Bcl-2 associated death promoter protein) which is an important mediator in the apoptotic pathway. Furthermore, it was reported that apoptosis mediated by β-amyloid is inhibited by activation of PI3K/Akt-signaling, as high levels of phosphorylated PI3K and Akt were observed after treatment of vitamin K2. Thus, PI3K/Akt/Bad-signaling pathway activation and inhibition of caspase-mediated apoptosis are the mechanisms underlying protective role of vitamin K2 as shown in (Huang et al., 2021) as shown in **Figure 5**.

Another factor that contributes to pathogenesis of Alzheimer's disease is overactivated microglia. When microglia are overactivated, they trigger inflammatory cascades in the central nervous system. Disruption of microglial homeostasis leads to activation of neurotoxic astrocytes, synaptic loss and neuronal death resulting in inflammation and neurodegeneration (Spangenberg et al., 2016; Liddelow et al., 2017). Saputra *et al.* investigated vitamin K2 (MK-4) effect on activation of microglia and the underlying mechanism. In MG6 cells derived from mouse microglia exposed to lipopolysaccharide, different concentrations of vitamin K2 (0–10 μM) were used; wherein the upregulation of inflammatory cytokines like *IL-1β*, *IL-6* and *TNF-α* were highly suppressed at 10 μM at mRNA level. Vitamin K2 inhibited the nuclear translocation of NF-κB in MG6 cells induced by LPS, resulting in inhibition of NF-κB signaling. Further, phosphorylation of p65 was suppressed significantly due to the presence of vitamin K2 but the levels of TAK1 and IKKα/β were unaffected (Saputra et al., 2019).

PHARMACOMETRICS OF VITAMIN K2-7

Clinical Pharmacokinetics of K2-7

ADME (absorption, distribution, metabolism, and excretion) kinetics are major factors that determine the safety and efficacy of K2-7. The absorption of K2-7 takes place from the lumen of ileum and jejunum. Absorption is rapid; K2-7 is incorporated into micelles or TAG-rich lipoproteins and doesn't undergo any change during the process (Beulens et al., 2013). Micelles are packed into chylomicrons secreted by enterocytes; chylomicrons are then secreted out from within the intestinal villi *via* exocytosis into lymphatic system through thoracic duct and reach the circulatory system. Changes take place in the apoproteins of chylomicrons that contain K2-7, these changes help in their uptake *via* endocytosis in the bone osteoblasts, liver, and other tissues; endocytosis is mediated by lipoprotein receptors such as, low-density lipoprotein receptor (LDLR) and low-density receptor-related protein (LRP). Excretion of K2-7 involves shortening of isoprenoid side chains to form metabolites of carboxylic acid with 5-carbon side chains. Further, conjugation of these metabolites with glucuronic acid takes place and finally they are excreted in urine and bile (Shearer et al., 2012). A study reported that K2-7 and other long chain derivatives of vitamin K2 are readily available for the extra-hepatic tissues like vasculature and bone after redistribution to the circulatory system (Schurgers and Vermeer, 2002).

In a study conducted by Schurgers *et al.* (Schurgers et al., 2007), MK-7 (1500 mg/L) in an oil solution form was mixed with vitamin K1 resulting in a mixture containing 100 μg/ml of vitamin K1 and K2 (MK-7) each. 10 ml of this mixture was emulsified with 20 ml orange juice and administered to 15 volunteers between the ages of 25–35 years. The peak serum concentration (C_{max}) of MK-7 was reported to be 17 μg/L and time required to achieve maximum concentration (T_{max}) was reported to be 4 h. The decline in MK-7 serum concentration was slow between 8 and 9 h and followed a biphasic pattern. The half-life ($t_{1/2}$) of MK-7 was reported to be 68 h. In a subsequent study, 10 volunteers received increasing doses (50, 100, 150, 200, 250, 300, and 500 μg each of the two vitamins) from a mixture of 100 μg/ml of vitamin K and MK-7. Both vitamins showed a linear dose response 4 h after mealtime. At 24 h after mealtime, the circulating concentration of MK-7 at 100 μg was reported to be approximately 1.5 nM (1 μg/L).

Sato *et al.* (Sato et al., 2012) investigated the bioavailability of menaquinone-7 in healthy Japanese women. Within 10 min post-breakfast consisting of 13–17 g fat, a single dose of MK-7 (420 μg) was given to five healthy female subjects. The single dose of MK-7 reached maximum serum concentration at 6 h after administration and the C_{max} was reported to be approximately

6.5 ng/ml. In a subsequent study, five healthy female subjects were administered with 60 μg of MK-7 daily after supper for 7 days. The serum level of MK-7 after consecutive administration for 7 days was reported to be 8.5 ng/ml thus leading to the conclusion that efficient absorption of nutritional dose of MK-7 is seen in humans with a significant rise in serum levels.

A study (Knapen et al., 2016) was performed in 107 volunteers in the Netherlands wherein postmenopausal women and healthy men (between 45 and 65 years of age) were subjected to a daily dose of *1*) MK-7-fortified yogurt (yogurt K), *2*) yogurt containing MK-7, magnesium, vitamin C and vitamin D3, fish oil and n-3 poly unsaturated fatty acids (n-3 PUFA) (yogurt Kplus) and *3*) soft gel capsules of MK-7, for 42 days. The MK-7 intake from either of the yogurts was reported to be 30.6 μg per serving while the soft gel capsules contained 58.3 ± 1.1 μg of MK-7. The participants were randomly assigned to receive either one soft gel capsule containing MK-7 or two servings of either yogurt K or yogurt Kplus daily for 42 days. The yogurt Kplus group was observed to have the highest plasma concentration during the initial 14 days of the treatment (2.75 ng/ml), while the plasma concentrations of yogurt K and capsules group in the same period were reported to be 2.5 and 2.25 ng/ml, respectively. Throughout the intervention of 42 days, the average plasma concentrations of the yogurt Kplus, yogurt K and capsule group were reported to be 2.29 ± 0.08 ng/ml, 2.17 ± 0.09 ng/ml, and 2.00 ± 0.09 ng/ml, respectively. Biphasic decline of MK-7 was observed during the 2-week washout period with a $T_{1/2}$ of 3 and 8 days during the first phase and second phase, respectively. The plasma levels of dephospho-uncarboxylated matrix Gla protein (dp-ucMGP) was decreased to 485 ± 30 pmol/L in the yogurt Kplus group, 417 ± 33 pmol/L in the yogurt K group and 434 ± 31 pmol/L in the capsules group. Yogurt Kplus group was reported to have the largest improvement of MGP carboxylation, however this effect was majorly attributed to the response in women.

Moller *et al.* investigated the bioavailability of synthetic MK-7 and fermented MK-7 by conducting a single blinded two-way cross-over study. Synthetic or fermentation-derived MK-7 (180 μg) were administered orally as a single dose to nine patients and eight patients respectively. It was observed that the 90% confidence interval for the ratio of the AUC (0–72 h) values for synthetic and fermentation-derived MK-7 was 83–111, indicating bioequivalence. The 90% confidence interval for C_{max} ratio was 83–131 (Møller et al., 2017). Due to the widespread use of MK-7-containing supplements, the pharmacokinetics following single intake of MK-7 from various formulations was assessed in four parallel studies. Participants were administered either tablets or capsules containing 180 μg MK-7. It was observed that T_{max} of tablet (6 h) was slower as compared to capsules (2–4 h). This can be attributed due to the oily matrix of the capsules that released MK-7 more rapidly than the tablet powder matrix. It was also observed that all the formulations gave similar 24 h absorption profiles which indicated that the vehicle carrier does not affect the absorption of MK-7 (Mhj, 2014).

Clinical Pharmacodynamics of K2-7

To estimate the effective dose of MK-7, Theuwissen *et al.* conducted a double blind, randomized, controlled study including 42 Dutch participants who were divided into seven groups and were administered with supplements (capsules) of placebo or different concentrations of MK-7 (10, 20, 45, 90, 180 or 360 μg) daily for 3 months. The plasma concentration range of MK-7 for the groups administered with 10, 20, 45, 90, 180 and 360 μg were reported to be 0–0.5, 0.4–2.1, 0–1.6, 0.4–4.6, 0.5–4.2, 1.1–6.5 and 4.1–10.6 ng/ml, respectively, thus indicating a direct correlation between plasma MK-7 concentration and MK-7 supplementation. Intake of 90 μg or more per day resulted in a significant increase in circulating concentrations as compared to placebo. Further, it was reported that MK-7 supplements at concentrations around recommended dietary allowance (RDA = 75 μg) (Theuwissen et al., 2012) enhanced carboxylation of matrix Gla protein (MGP) and osteocalcin (OC), while a decrease in the levels of circulating dephospho-uncarboxylayed MGP (dp-ucMGP) and uncarboxylated osteocalcin (ucOC) as well as in the ucOC:cOC ratio was observed. Following the highest intake of MK-7, the level of circulating cOC was significantly increased. No significant effects were observed on the circulating levels of osteocalcin and MGP at supplementation doses of MK-7 below RDA.

Knapen *et al.* (Knapen et al., 2018) conducted a randomized, placebo-controlled study including 214 postmenopausal women between the age group 0f 55–65 years of age wherein they received either 180 μg/day of vitamin K2 (MK-7) or placebo for 3 years. It was observed that MK-7 increased the levels of circulating osteocalcin (21.5%) as compared to placebo (2.6%) however there was no significant effect of MK-7 on body fat or fat distribution. In MK-7 group, levels of undercarboxylated osteocalcin (ucOC) were decreased by 50.1%, while in placebo group, no significant change was observed (+4.3%). In subjects that responded well with respect to osteocalcin carboxylation (good responders), MK-7 was reported to increase total and human molecular weight adiponectin, while abdominal fat mass and estimated visceral adipose tissue area were reported to be decreased as compared to poor responders as well as placebo. In good responders, the change in carboxylated osteocalcin was 1.66 ng/ml or 34.9% and in poor responders it was 0.38 ng/ml or 7.7%.

Biological effects of fermentation derived MK-7 (90 μg) and 3 doses of synthetic MK-7 (45, 90, and 180 μg) were compared in a randomized double-blinded parallel study. Placebo, 45 μg synthetic MK-7, 90 μg synthetic MK-7, 180 μg synthetic MK-7 or 90 μg fermentation-derived MK-7 were administered daily to the subjects for 43 days. Blood samples were collected for measurement of MK-7 as well as serum concentrations of cOC and ucOC. There was an increase in serum cOC concentration and a reduction in serum ucOC concentration after daily intake of the highest dose of synthetic MK-7 (180 μg) for 6 weeks. The fermentation-derived MK-7 group showed similar results. Therefore, it was concluded that the tested synthetic form of MK-7 is bioequivalent to fermentation-derived MK-7, exhibits vitamin K activity and can be well tolerated in healthy subjects (Møller et al., 2017).

A study was carried out by Aoun *et al.* to determine the level of dp-ucMGP, after treatment using MK-7 in haemodialysis patients. It is known that dp-ucMGP increases in vitamin K-deficient patients which can be associated with vascular calcification. Hence, 50 haemodialysis patients from Eastern Mediterranean cohort were administered 360 μg of MK-7 for 4 weeks. The results indicated 86% decrease of dp-ucMGP after 4 weeks of treatment; however, further studies are required to assess the change of vascular calcification after longer duration of treatment (Aoun et al., 2017). Also, a randomized dose-finding study was performed in 200 chronic hemodialysis patients. Patients were administered MK-7 with either 360, 720 or 1080 μg thrice weekly for 8 weeks. It was observed that decrease in dp-ucMGP was 17, 33 and 46% respectively indicating a dose dependent response by MK-7 supplementation. Few side-effects that were reported were mild and independent of the dose (Caluwe et al., 2014). Likewise, a randomized trial was carried out in 53 hemodialysis patients in which 45, 135 or 360 μg of MK-7 was administered daily for 6 weeks and compared with 50 healthy patients (control group). After 6 weeks of treatment, the plasma levels of the hemodialysis patients were assessed which indicated the level of dp-ucMGP and ucOC was 4.5- and 8.4-fold respectively higher than the control group. Forty-nine patients showed elevation in the protein induced by vitamin K absence II (PIVKA-II). It was also observed that MK-7 supplementation showed a dose- and time-dependent response in dp-ucMGP and ucOC and PIVKA-II levels (Westenfeld et al., 2012).

An experiment was performed by Brugè *et al.* to study the effect of K2-7 supplementation with extra-virgin olive oil on carboxylation of OC. Olive oil enriched with 45 or 90 μg of K2-7 was administered for 2 weeks in 12 healthy volunteers in Italy. The results demonstrated significant increase in cOC:ucOC ratio with 90 μg of K2-7 which improves bone mineralization; however, olive oil with 45 μg K2-7 did not show a biological effect. Hence, it was concluded that extra-virgin olive oil enriched with K2-7 can improve bone mineralization (Brugè et al., 2011). Dalmeijer *et al.* (Dalmeijer et al., 2012) conducted a randomized double-blind placebo-controlled study for 12 weeks to investigate the effect of K2-7 on carboxylation of matrix Gla protein (MGP). Sixty patients that were administered with 180 or 360 μg showed dose-dependent decrease in dp-ucMGP; however, no change was observed in dp-cMGP and total ucMGP level (Dalmeijer et al., 2012).

A randomized controlled trial was conducted by Sakak et al. (Rahimi Sakak et al., 2021) on 68 patients with type 2 diabetes on oral glucose-lowering therapy. Patients were either administered 360 μg of MK-7 or placebo daily for 12 weeks. No significant difference in the atherogenic status between the MK-7 or placebo group was observed. Hence, it was concluded that 360 μg of MK-7 supplementation for 12 weeks cannot improve the insulin resistant (IR)-related indexes of cardiovascular risk (Rahimi Sakak et al., 2021). Also, a randomized, double-blind placebo-controlled trial was performed to assess whether the supplementation of menaquinone-7 decreases vascular calcification in patients having type 2 diabetes and cardiovascular disease (CVD). Sixty-eight patients were randomly administered either with 360 μg daily of MK-7 or placebo for 6 months. Blood samples of the patients administered with MK-7 showed increased active calcification compared with placebo when measured with ^{18}sodium fluoride positron emission tomography (^{18}F-Na PET) activity; however, no effect was found in calcification mass on conventional computed tomography (CT) (Zwakenberg et al., 2019).

Inaba *et al.* investigated the effective minimum daily intake of MK-7 to improve osteocalcin γ-carboxylation. In postmenopausal Japanese women aged 50–69 years, a significant increase was observed in the ratio of osteocalcin/undercarboxylated osteocalcin and decrease in ucOC concentration when 100 and 200 μg was administered daily for 4 weeks of MK-7. Similar results were obtained in healthy males, and females aged 20–69 years in 100 μg daily intake of MK-7 for 12 weeks (Inaba et al., 2015). A randomized double-blind placebo-controlled trial was carried out in 334 early postmenopausal women. 360 μg of MK-7 in the form of natto capsules was administered daily for 1 year. Serum level showed significant improvement in cOC levels; however, there was no improvement in the bone mass density (BMD) in total hip, femoral neck, lumbar spine and total body. This suggested that there was no improvement in the bone loss rates after the 1 year treatment (Emaus et al., 2010).

The effect of MK-7 supplementation on the activity of vitamin K-dependent procoagulant factors was investigated by Ren et al. (Ren et al., 2021). Forty healthy volunteers were administered 90 μg of menaquinone-7 for 30 days; and prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), blood coagulation factors II, VII, IX and X activities and protein induced by vitamin K absence or antagonist-II (PIVKA-II) were measured in blood samples. The results indicated that supplementation of MK-7 at the recommended dose does not affect the vitamin K dependent coagulation factors (Ren et al., 2021). In a clinical study, Zhelyazkova-Savova *et al.* (Zhelyazkova-Savova et al., 2021) examined the effects of statin drug on vitamin K2 status and VKDPs. It was observed that the levels of uncarboxylated osteocalcin (ucOC) and ucOC:cOC ratio increased in patients that were administered with statin. In addition, statin inhibited activity of VKDPs and calcium accumulation was increased in arterial walls. A study conducted by Zhang et al., 2012. determined the synergistic effect of sorafenib and vitamin K2 on human hepatocellular carcinoma (HepG2) cells. *In vitro*, co-administration of 2.5 μM sorafenib with 1 μM vitamin K2 synergistically inhibited the HepG2 cell proliferation. *In vivo* study was carried out in nude mice by inoculation of EGFP-expressing HepG2 cells. Sorafenib (1.25 mg/kg body weight) and vitamin K2 (2 mg/kg body weight) were injected daily for 18 days. It was observed that sorafenib and vitamin K2 inhibited the tumor growth synergistically in combination therapy. In a study conducted by Hara et al. in rat aorta loop model, it was observed that when vitamin K2 (1.5, 14 and 145 mg/kg) and warfarin (0.80 mg/L) were administered simultaneously, the high dose of K2 (145 mg/kg) reduced the effect of warfarin on thrombosis suggesting on interaction between K2 and warfarin (Hara et al., 1999). A study was carried out by Theuwissen et al. to determine the effect of MK-7 supplementation on vitamin K antagonist therapy (VKA). Eighteen healthy volunteers were administered with increasing doses (10–20–45 μg) of MK-7 for 6 weeks after previous treatment with acenocoumarol for 4 weeks. The study reported that MK-7 supplementation as low as 10 μg should be avoided during VKA as it can significantly affect the anticoagulation sensitivity in few

TABLE 3 | Clinical trials on vitamin K2-7 in the United States of America (Adapted from www.clinicaltrials.gov).

Clinical trial number	Status	Phase	Number of participants	Conditions or disease	Objective	Dose	Reference
NCT04770740	Recruiting	II	40	COVID-19	To study the effect of vitamin K2-7 in COVID	K2-7: 333 µg/day for 14 days Placebo: 3 tablets/day for 14 days	Dofferhoff, (2021)
NCT02839044	Completed	NA	68	Arterial calcification, Type 2 Diabetes mellitus (T2DM)	To investigate the influence of vitamin K2	K2-7: 360 µg/day Placebo: Tablets daily	Schouw, (2019)
NCT00165633	Terminated	II/III	540	Hepatocellular carcinoma	To detect the inhibitory effects on recurrence	Menatetrenone (E0167): 45/90 mg capsule thrice daily	Eisai Limited, (2008)
NCT00931437	Completed	NA	12	Healthy	To study the absorption from dairy	Vitamin K-rich dairy products	Maastricht University Medical Center, (2010a)
NCT03360435	Completed	NA	99	Post bariatric surgery patients	To study absorption of transdermal vitamins	Multivitamins	University of Florida, (2021)
NCT02917525	Unknown	II	44	Bicuspid aortic valve stenosis	To study the effect on calcium metabolism	360 µg vitamin K2 daily for 18 months Placebo for 18 months	Maastricht University Medical Center, (2018a)
NCT00642551	Completed	NA	240	Bone loss Arteriosclerosis	To investigate long term beneficial effects	180 µg K2-7 daily for 3 years 1 placebo capsule daily for 3 years	Maastricht University Medical Center, (2012a)
NCT04641663	Recruiting	NA	70	Aging or age-related cognitive decline	To detect tolerability towards multi target dietary supplement (MTDS)	MTDS: Morning (five tabs), Evening (three tabs) and OMEGA (two softgels)	Boreham, (2021)
NCT04477811	Completed	II/III	40	Renal disease	Comparative study for vitamin K1 and K2	10 mg vitamin K1 thrice/week for 3 months 90 µg vitamin K2 per day orally	Farid, (2021)
NCT01873274	Completed	NA	107	Bioavailability	Comparative study for different delivery systems	Basic yogurt enriched with MK-7 (50 µg) MK-7 containing capsule (50 µg) Nutrient-enriched yogurt with MK-7	Maastricht University Medical Center, (2013)
NCT00858767	Completed	NA	69	Bioavailability	To examine arabic gum absorption	MK-7 from casein, arabic gum and linseed oil capsules	Maastricht University Medical Center, (2010b)
NCT00742768	Completed	NA	16	Healthy	Comparative study	Vitamin K2 in softgel Gelpell	Maastricht University Medical Center, (2018b)
NCT01638143	Completed	NA	24	Bioavailability	Bio-equivalence	Gnosis P-1000 capsule Gnosis M-1500 capsule MenaQ7 M-1500 capsule	Maastricht University Medical Center, (2012b)
NCT01638182	Completed	NA	81	Bone and vascular health	Bio-comparison	52 µg of vitamin K1 75 µg of vitamin K2 for 3 months 1 placebo/day for 3 months	Maastricht University Medical Center, (2012c)
NCT01194778	Completed	NA	82	Carboxylation level vitamin K-dependent proteins	To compare the efficacy	1 placebo sachet per day containing only sucrose for 12 weeks 15 µg vitamin K1 per day for 12 weeks 15–45 µg (15 µg interval) vitamin K2 per day during 12 weeks for three groups	Maastricht University Medical Center, (2010c)
NCT04382027	Completed	NA	20	Healthy	To study pharmacokinetics of omega-3 with vitamin K2	Omega-3 + vitamin K2 (monoacylglycerol form) Omega-3 + vitamin K2 (ethyl ester)	Plourde, (2020)
NCT03305536	Unknown	II	150	Aortic valve disease	To investigate decalcification of aortic valve	Vitamin K2 (1000 µg/day) + vitamin D3 (5000 IU/day)	Frangi, (2019)
NCT04780061	Recruiting	III	200	COVID-19	To use vitamin K2 as dietary supplement	Combination of vitamin K2 (30 µg) and vitamin D2 (3.125 µg)	The Canadian College of Naturopathic Medicine, (2021)

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TABLE 3 | (*Continued*) Clinical trials on vitamin K2-7 in the United States of America (Adapted from www.clinicaltrials.gov).

Clinical trial number	Status	Phase	Number of participants	Conditions or disease	Objective	Dose	Reference
NCT00483431	Completed	NA	42	Vitamin K-status	Dose finding	MK7 10 µg (1 cap) + 3 placebo capsules MK7 20 µg (2 cap) + 2 placebo-capsules MK7 45 µg (1 cap) + 3 placebo-capsules MK7 90 µg (2 cap) + 2 placebo-capsules MK7 180 µg (4 capsule) MK7 360 µg (1 cap) + 3 placebo-capsules All above doses taken daily for 12 weeks	Maastricht University Medical Center, (2018c)
NCT04189796	Not yet recruiting	NA	64	Healthy women osteocalcin	Carlsberg vs camembert cheese	Oral intake of both cheese for 6 months	Larsen, (2019b)
NCT04041492	Completed	NA	100	Type 2 Diabetes mellitus, Bone Loss, Insulin resistance	To study effects of vitamin K2	Supplementation of vitamin K2 with vitamin D3 one capsule every 24 for 3 months	Ruiz, (2019)
NCT02976246	Completed	IV	123	Renal disease, Cardiovascular disease, Bone disease	To study effects of vitamin K2	1 tablet of MK-7 360 µg once daily 1 tablet of placebo once daily	Zealand University Hospital, (2020)
NCT04539418	Completed	IV	59	Renal disease	To determine effects of K2 on vascular calcification	Vitamin K2: thrice/week at the end of each dialysis session through the ultrafiltration membrane intravenously	Abúd, (2020)
NCT04010578	Not yet recruiting	NA	52	Coronary artery disease (CAD), Carotid artery disease	To study effects of vitamin K2 and vitamin D3 on CAD	400 µg of MK7 and 80 µg of vitamin D3/day Daily placebo for 3 months	Mottaghy, (2021)
NCT04676958	Recruiting	NA	80	Inflammation, Oxidative Stress, Exercise, Strength, Recovery	To examine recovery of vitamin K2 from exercise	380 mg capsule/day micro-crystalline cellulose with 240 µg/day vitamin K2	Gray, (2021)
NCT01143831	Completed	NA	8	Thrombosis	Evaluation of proteins C and G; and factors II, VII, IX, X	Vitamin K2 in 5 mg capsules for 14 days	MD, (2012)
NCT01407601	Completed	III	53	Chronic kidney disease (CKD) Stage 5D, Hemodialysis	To activate matrix Gla protein (MGP)	45 µg, 135 and 360 µg of MK-7 daily prior to dialysis over 6 weeks	RWTH Aachen University, (2011)
NCT01101698	Unknown	IV	60	Kidney disease, Coronary artery calcification	To study the influence of vitamin K2	90 µg vitamin K2 with 10 µg vitamin D once daily for 9 months	Medical University of Lodz, (2010)
NCT04429035	Recruiting	NA	200	Aortic valve calcification and stenosis, Mitral annular calcification, Mitral valve calcification and stenosis	To slow down progress of calcification	300 µg vitamin K2 daily orally 300 µg vitamin K2 and placebo daily orally	Toutouzas, (2021)
NCT02404519	Unknown	NA	240	Vascular stiffness	To study effect of vitamin K2	180 µg K2-7 daily orally for 1 year 1 Placebo tablet daily orally for 1 year	Knapen, (2018)
NCT01922804	Unknown	NA	150	Metabolic bone disorder	To study effect on bone and glucose metabolism	Vitamin K2 375 µg/day for 3 years 1 Placebo tablet/day for 3 years	University of Aarhus, (2016)
NCT01870115	Completed	I	23	Osteopenia	To investigate effect of melatonin and micro-nutrients	2.5 mg melatonin, 225 mg strontium citrate, 1000 IU vitamin D3 and 30 µg vitamin K2	Duquesne University, (2018)
NCT01923012	Unknown	II	200	Carotid disease, Atherosclerosis	To determine effect of vitamin K2	Vitamin K2	Vidale, (2014)
NCT00290212	Completed	II	304	Perimenopausal bone loss	To investigate prevention of bone loss	Natto capsules + 360 µg vit K2/day	University Hospital of North Norway, (2008)

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TABLE 3 | (*Continued*) Clinical trials on vitamin K2-7 in the United States of America (Adapted from www.clinicaltrials.gov).

Clinical trial number	Status	Phase	Number of participants	Conditions or disease	Objective	Dose	Reference
NCT04428606	Recruiting	NA	60	Pre-diabetes	To determine effect of synbiotics	Metabolic Rheostat™ (2 capsules 3× per day for 56 days) Butyrate Ultra (2 capsules 3× per day for 56 days) Placebo (2 capsules 3× per day thrice for 56 days)	Li, (2021)
NCT01675206	Completed	III	165	Vascular calcification	Dose defining study	360 µg, 720 and 1080 µg of vitamin K2 thrice weekly	Caluwe, (2012)
NCT03871322	Recruiting	NA	90	Fracture healing	To determine effects of vitamin D3 and K2 on fracture healing	Supplementation of vitamin D and K2	Medical University of Bialystok, (2021)
NCT04387019	Completed	-	90	Type 2 Diabetes mellitus	To study blood samples	Diagnostic test (samples from peripheral veins)	Helmy, (2021)
NCT02876354	Completed	IV	50	Vascular calcification	To evaluate risk factors	Menaquinone 360 µg /day for 4 weeks	Aoun, (2016)
NCT02970084	Completed	NA	60	Carotid plaques	ffect on calcium levels	Nutraceutical product based on vitamin K2, once a day (a tablet 800 mg) for 12 months	Tshomba, (2019)
NCT03799822	Completed	IV	132	Atrial fibrillation	Anti-coagulation	Rivaroxaban (10 mg) oral tablet + vitamin K2 (2000 µg) thrice weekly	Caluwe, (2020)
NCT00512928	Completed	NA	20	Healthy	To determine safety of vitamin K2 during anticoagulation	10–20 µg increasing to 45 µg for final week	Maastricht University, (2008)
NCT00548509	Completed	IV	131	Osteoporosis	To study the effect of K2 on bone turnover	Menatetrenone (Vitamin K2) therapy	Eisai Limited, (2018)
NCT01533441	Completed	II	50	Thrombosis	Intervention with antagonists of vitamin K (VKA)	4 months dairy product + treatment (vitamin K2 or placebo)	Danisco, (2014)
NCT02610933	Completed	IV	117	Vascular calcification	Replacement of VKA by Rivaroxaban + vitamin K2	Rivaroxaban (10 mg) tablet once daily and MK-7 (2000 µg) tablet thrice weekly for 18 months	Caluwe, (2019)
NCT03243890	Completed	NA	389	Aortic valve stenosis	Decalcification	720 µg/day MK7 and vitamin D (25 µg/day)	Diederichsen, (2021)
NCT01002157	Unknown	NA	180	Coronary artery disease (CAD)	Effect on progression of coronary artery calcification	Vitamin K2 Placebo control (containing no vitamin K2)	Maastricht University Medical Center, (2018d)
NCT01928134	Unknown	NA	60	Healthy	Efficacy of vitamin K2	Vitamin K2 + Vitamin D3 + calcium carbonate ($CaCO_3$)	Su, (2013)
NCT04145492	Unknown	II/III	60	End stage renal disease (vascular calcification)	Effect of vitamin K2	90 µg of vitamin K2-7 daily + standard therapy for 4 months Vitamin K2-7 (90 µg) + 10 µg of vitamin inactive vitamin D daily + standard therapy for 4 months	Borolossy, (2019)
NCT00402974	Completed	NA	55	Healthy	Effect on osteocalcin carboxylation in children	45 µg vitamin K2 for 8 weeks	UMC Utrecht, (2008)
NCT02870829	Active, not recruiting	II	178	Systemic and arterial stiffness complication of hemodialysis	Reduction of vascular calcification	Oral supplement of MK7 post dialysis thrice/week	Peixin, (2020)
NCT02366481	Active, not recruiting	NA	30	Obesity, Insulin resistance, Insulin sensitivity, β-cell dysfunction, Pre-diabetes	Effect on glucose metabolism in adults	Low dose: 1 vitamin K2 (90 µg) and 1 placebo softgel capsule daily for 8 weeks High dose: 2 vitamin K2 (90 µg) daily for 8 weeks	Pollock, (2019a)

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TABLE 3 | (*Continued*) Clinical trials on vitamin K2-7 in the United States of America (Adapted from www.clinicaltrials.gov).

Clinical trial number	Status	Phase	Number of participants	Conditions or disease	Objective	Dose	Reference
NCT01972113	Recruiting	NA	30	Obesity, Insulin resistance, Insulin sensitivity, β-cell dysfunction, Pre-diabetes	Effect on glucose metabolism in children	Low dose: 1 vitamin K2 (45 μg) and 1 placebo softgel capsule daily for 8 weeks High dose: 1 vitamin K2 (90 μg) daily for 8 weeks	Pollock, (2019b)
NCT04900610	Not yet recruiting	NA	120	Peritoneal dialysis	Effect on arterial stiffness and cardiovascular events	Vitamin K2 1 mg/day orally	Vasileios, (2021)
NCT03311321	Recruiting	NA	60	Cardiovascular disease (CVD), Chronic kidney disease (Stage 3, 4 and 5)	Slow down progression of CVD risk in hemodialysis patients	4 vitamin K2 (90 μg) softgel capsules daily for 8 weeks 4 placebo softgel capsules daily for 8 weeks (without vitamin K2)	Pollock, (2019c)
NCT02959762	Active, not recruiting	NA	30	Insulin resistance, Obesity in diabetes, Nutritional and metabolic diseases, Hyperlipidemia, Hyperglycemia	Slow down progression of Dyslipidemia and diabetes risk in children	Placebo control for 8 weeks Low dose: 1 vitamin K2 (45 μg) and 1 placebo softgel capsule daily for 8 weeks High dose: 2 vitamin K2 (45 μg) daily for 8 weeks	Pollock, (2019d)
NCT02517580	Completed	II	60	Arterial stiffness	Effect on arterial stiffness in the renal transplant population	Vitamin K2 (360 μg/day) once daily for 8 weeks	Ph.D, (2017)
NCT03493087	Completed	NA	33	Renal Insufficiency, Chronic	Why hemodialysis patients have a low vitamin K status and how to improve it	MK-7 360 μg tablet /day for 6 weeks Diet rich in vitamin K for 6 weeks	NCT03493087. (2019)
NCT04669782	Active, not recruiting	NA	123	Bone Metabolism Disorder, Aging Disorder and, Osteoporosis	Effect on bone and skeletal muscle, and energy metabolism in patients with severe osteoporosis	MK-7 (375 μg/day) + Teriparatide Teriparatide will be administered as standard	University of Milano Bicocca, (2020)
NCT04285450	Recruiting	IV	99	Diabetes Mellitus, Type 2	Effect on the glycemic control, insulin sensitivity and lipid profile	Vitamin K 1 mg Placebo	Mohamed, (2020)
NCT03897660	Completed	NA	22	Healthy	Better bioavailability of Omega-3 and Vitamin K2	Omega-3 + vitamin K2 (TG form of omega-3) Omega-3 + vitamin K2 (EE form of omega-3) Omega-3 + vitamin K2 [MaxSimil (MAG form of omega-3)]	Plourde, (2020)
NCT03813550	Unknown	NA	20	Pseudoxanthoma Elasticum (PXE)	Association between gut microbiota composition, plasma levels of vitamin K and severity of clinical manifestations in PXE patients	Not Applicable	University Hospital, Angers, (2019)
NCT04188080	Unknown	NA	12	Maintenance dose, Osteocalcin, Vitamin K deficiency	To estimate the maintenance of daily dose of Jarlsberg cheese	Maintaining daily dose of Jarlsberg cheese	Larsen, (2019a)
NCT04612088	Active, not recruiting	NA	34	Bariatric patients	To promote multivitamin adherence	Bariatric Multivitamin (two tablets once a day for 3 months)	Sanchez, (2021)

TABLE 4 | Other global clinical trials on vitamin K2-7.

Clinical trial number	Status	Phase	Number of participants	Conditions or disease	Objective	Dose	Reference
ISRCTN18436190	Completed	NA	95	Anteroposterior sway	To improve balance and reduce the risk of falls in older people	Oral vitamin K2 (200 µg or 400 µg once daily for 1 year)	ISRCTN - ISRCTN 18436190. (2018)
ISRCTN21444964	Completed	NA	159	Chronic kidney disease stages 3B and 4	To study effect on pulse wave velocity	400 µg once daily oral vitamin K2 (MK7 subtype) vs matching placebo	ISRCTN - ISRCTN21444964. (2015)
ISRCTN93213492	Completed	NA	80	Vascular disease	To study effect of vitamin K supplementation on markers of vascular health in older people	Vitamin K2 (MK7 subtype) 100 µg per day or placebo	ISRCTN - ISRCTN93213492. (2012)
NL5147	Unknown	NA	NA	Diabetes patients, arterial calcification, bone metabolism	To reduce ongoing calcification and significantly reduce dephosphorylated-uncarboxylated mat dp-ucMGP	Vitamin K2 (menaquinone-7)	NTR NL5147. (2016)
NL1338	Open for patient inclusion	NA	140	Postmenopausal women	To reduce osteoclast activity and increase carboxylation of osteocalcin and MGP	Dairy product low vitamin K Dairy product + 100 µg vitamin K1 Dairy product + 100 µg vitamin K2	NTR NL1338. (2009)
NL1876	Planned	NA	60	CVD, Atherosclerosis	Vitamin K2 can increase the carboxylation of the matrix Gla protein (MGP)	Two groups will receive a daily dose of 180 or 360 µg vitamin K2 respectively for 12 weeks Placebo group: 1 capsule daily	NTR NL 1876. (2011)
NL9013	Planned	NA	20	Cardiovascular disease	To reduce the risk of coronary calcification	1 sachet of probiotics producing vitamin K2 for 12 weeks Placebo	NTR NL9013. (2021)
NL7296	Planned	NA	20	Vitamin K deficiency, Cardiovascular risk	To improvise vitamin K status	A daily sachet of 4 gm dried multispecies probiotics or placebo	NTR NL7296. (2020)
DRKS00016046	Completed	NA	30	Healthy	To study efficacy of topical application of fat-soluble vitamins in liposomes	Vit A (780 IU) + Vit D (1500 IU) + Vit E (1.38 IU) + Vit K2 (66 µg), Vit A (1560 IU) + Vit D (3000 IU) + Vit E (2.77 IU), Vit K2 (133 µg) Vit A (3120 IU), Vit D (6000 IU), Vit E	DRKS00016046. (2018)

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TABLE 4 | (*Continued*) Other global clinical trials on vitamin K2-7.

Clinical trial number	Status	Phase	Number of participants	Conditions or disease	Objective	Dose	Reference
						(5.54 IU), Vit K2 (265 μg) for 3 weeks (blood draw in the last 3 days of the intervention)	
jRCTs051180115	Recruiting	NA	200	Hepatocellular carcinoma (HCC)	TACE (transcatheter arterial chemoembolization) + vitamin K treatment	Daily dosing of vitamin K2 (45 mg) for 1 day before TACE to 28 days after	JRCTs051180115. (2019)
UMIN000033832	Pending	NA	15	Healthy adult	To study osteogenesis by intake of vitamin K2-rich natto	Natto	UMIN000033832. (2018)
UMIN000030521	Complete: follow-up continuing	NA	10	Warfarin treated patients	To evaluate PT-INR (Prothrombin Time-International Normalized Ratio)	Warfarin-treated patients who eat low vitamin K2 Natto for 7 days. When 10 g is acceptable, same patients will take 20 g Natto	UMIN000030521. (2018)
IRCT20190824044592N1	Recruitment complete	III	68	Type 2 diabetes	To study effect on glycemic control and lipid profile	Vitamin K2 (180 μg capsule twice a day for 12 weeks) Placebo capsule twice daily for 12 weeks	IRCT. (2020)
IRCT201506185623N45	Recruitment complete	III	66	Type 2 diabetes and CVD	To determine effects of combined calcium, vitamins D and K supplementation	Vit D (200 IU) + Vit K2 (90 μg) + calcium (500 mg) twice a day for 12 weeks Placebo twice a day for 12 weeks	IRCT. (2019a)
IRCT20100123003140N22	Recruitment complete	III	46	Type 2 Diabetes	To study effect on metabolic, nutritional, inflammatory, and matrix γ-carboxyglutamate protein	Menaquinone supplement (200 g/day) Placebo (for 3 months)	IRCT. (2019b)
IRCT2017092836204N2	Recruitment complete	II / III	84	Polycystic ovary syndrome (PCOS)	To investigate effect on body mass index (BMI), waist circumference (WC), fasting blood sugar (FBS), lipid profile, endocrine markers and MDA	1 capsule of menaquinone per day Placebo	IRCT. (2022)

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TABLE 4 | (*Continued*) Other global clinical trials on vitamin K2-7.

Clinical trial number	Status	Phase	Number of participants	Conditions or disease	Objective	Dose	Reference
IRCT20170916036204N5	Recruitment complete	NA	84	Polycystic ovary syndrome (PCOS)	Effect on depression status, fasting glucose and serum vitamin K level in patients with PCOS	1 menaquinone capsule (90 μg) per day Placebo	IRCT. (2021)
IRCT201509015623N51	Recruitment complete	III	60	PCOS	Effects of vitamins D, K and calcium supplementation on metabolic profiles in women with PCOS	Vitamin D + Vitamin K + Calcium Placebo	IRCT. (2018)
ACTRN12619000170123	Completed	NA	125	Mild hypercholesterolemia cardiovascular diseases	Effect on lipid profile	BruMeChol™ twice/day for 12 weeks	ACTRN12619000170123. (2020)
ACTRN12619000102178	Completed	NA	88	Hypercholesterolemia Mixed dyslipidemia	Effect on lipid profile, oxidative stress and inflammation	Octacosanol (20 mg) + Vitamin K2 (45 μg)	(ACTRN12619000102178. (2021))
2019–004906–88	Ongoing	III	40	Cardiovascular diseases	Effect on vascular calcification	Vitamin K2 MK-7 capsule (360 μg)	Clinical Trials Register. (2019)
CTRI/2016/11/007499	Completed	NA	100	Type 2 diabetes mellitus Vitamin B12 deficiency	Effect of Vitamin K2 supplementation	Vitamin K2-7 capsule (100 μg) twice a day for 8 weeks	CTRI/2016/11/007499. (2017)
CTRI/2013/09/003998	Completed	NA	20	Idiopathic muscle cramps	Effect of Vitamin K2 supplementation in Idiopathic muscle cramps	Vitamin K2-7 capsule (100 μg) twice a day for 3 months	CTRI/2013/09/003998. (2021)
CTRI/2012/08/002930	Completed	NA	30	Megaloblastic Anaemia, Type 2 diabetes mellitus	Effect of Vitamin K2 supplementation	Vitamin K2-7 capsule (100 μg) twice a day for 8 weeks	CTRI/2012/08/002930. (2017)
CTRI/2017/09/009660	Completed	NA	16	Healthy	Bioavailability study of vitamin K2	Vitamin K2-7 (1000 μg)	CTRI/2017/09/009660. (2017)
CTRI/2017/06/008925	Completed	NA	60	Vitamin B12 deficiency, T2DM	Effect of Vitamin K2 supplementation	Vitamin K2-7 capsule (100 μg) twice a day for 8 weeks Placebo	CTRI/2017/06/008925. (2017)
CTRI/2018/05/014246	Completed	NA	80	Type 2 Diabetes mellitus with Hypertension	Evaluation of plasma levels of Vitamin K2-7	NA	CTRI/2018/05/014246. (2021)
CTRI/2019/03/018278	Completed	NA	50	Healthy	Evaluation of plasma levels of Vitamin K2-7	NA	CTRI/2019/03/018278. (2019)
CTRI/2019/06/019548	Completed	NA	20	Type 2 diabetes mellitus and Vitamin B12 deficiency	Plasma levels of Vitamin K2 upon oral supplementation	Vitamin K2-7 capsule (100 μg) twice a day for 8 weeks Placebo	CTRI/2019/06/019548. (2020)
CTRI/2019/12/022361	Completed	NA	15	Healthy	Pharmacokinetics of Vitamin K2-7	Vitamin K2-7 capsule (350 μg) for 21 days	CTRI/2019/12/022361. (2021)

(Adapted from ISRCTN, International Standard randomized Controlled Trial Number (www.isrctn.com); NL, Netherlands Trial Register (www.trialregister.nl); DRKS, German Clinical Trials Register (www.drks.de); jRCT, Japanese Registry for Clinical Trials (https://jrct.niph.go.jp/) UMIN, University hospital Medical Information Network (UMIN) Clinical Trials Registry (www.umin.ac.jp); IRCT, Iranian Registry of Clinical Trials (www.irct.ir); ACTRN, Australian Clinical Trials Registration Number (www.australianclinicaltrials.gov.au); EUCTR, European Union Clinical Trials Register (www.clinicaltrialsregister.eu); CTRI, Clinical Trials Registry- India (www.ctri.nic.in)).

individuals (Theuwissen et al., 2013). Further, a study was designed to determine the effective role of MK-7 in the therapeutic management of rheumatoid arthritis (RA). Eighty-four patients undergoing RA treatment were either administered 100 μg dose of MK-7 capsules or kept naïve without changing any other medication for 3 months. The clinical and biochemical markers like ucOC, erythrocyte sedimentation rate (ESR), disease activity score assessing 28 joints with ESR (DAS28-ESR), C-reactive protein and matrix metalloproteinase (MMP-3) were assessed using patients' serum. There was a significant decrease in clinical and biochemical markers such as ucOC, ESR and DAS28-ESR for moderate and good responders compared to non-responders. The results indicated that there was improvement in disease activity score of RA by changing the bone mineral metabolism when treated with MK-7 (Abdel-Rahman et al., 2015). Ozdemir et al. conducted a pilot study to investigate the therapeutic effect of dietary supplement of vitamin D (5 μg calcitriol) and vitamin K2 (50 μg MK-7) in 20 children on thalassemic osteopathy (TOSP). The serum samples collected after 6 and 12 months showed decrease in the ratio of ucOC to cOC; however, it was not significant (Ozdemir et al., 2013). Summeren et al. conducted a double blind randomized placebo-controlled trial to determine the effect of MK-7 supplementation on 55 pre-pubertal children aged between 6 and 10 years from Netherlands. 45 μg of MK-7 was administered daily for 8 weeks and compared with placebo group. The serum samples showed increase in MK-7 and decrease in circulating ucOC concentration (van Summeren et al., 2009).

Clinical Trials on K2-7

Several clinical trials have been performed to determine the effect of vitamin K2-7 on various diseases and conditions that include bone disease, renal disease, diabetes mellitus type 2, thrombosis, *etc*. Studies also include the determination of effect of vitamin K2-7 on patients suffering from COVID-19. Various clinical trials for determination of the appropriate dose of vitamin K2 have been performed; also its bioavailability and efficacy have been investigated in a few trials. Further, in many trials, vitamin K2-7 is combined with vitamin D as an intervention to augment the beneficial effects. Vitamin K2-7 is taken in the form of drug (capsules) or included in dietary supplements such as dairy products (yoghurt, cheese, *etc*). These have been summarized in **Table 3** and **Table 4** for the better understanding of the reader.

CONTRAINDICATIONS TO VITAMIN K2-7

There are no severe adverse effects due to the supplementation of vitamin K2-7. However, high doses of vitamin K2 can cause allergic reactions (Korah et al., 2017). In a study conducted to find the effect of K2-7 on warfarin on thrombotic rate using a rat aorta model, it was observed that high dose of vitamin K2-7 (145 mg/kg) reduced the effect of warfarin (0.80 mg/L) on thrombosis (Hara et al., 1999). There are no other known major contraindications to K2-7 intake as of current knowledge.

CONCLUSIONS

Vitamin K2-7 or menaquinone-7, a naturally occurring form of vitamin K2, is easily absorbed and readily bioavailable compared to other forms. Supplementation of the diet with K2-7 has health-beneficial effects in various diseases including osteoporosis, cardiovascular diseases, cancer, diabetes and related complications, and neurodegenerative diseases. The molecular underpinnings of these beneficial effects involve complex regulatory crosstalk between important signal transduction cascades in the cellular milieu. It can also potentially involve the interplay of noncoding RNAs such as miRNAs and lncRNAs which merit further investigation. Clinical evidence from our research group has unequivocally demonstrated the utility of K2-7 dietary supplementation for peripheral neuropathy. Taken together, the health-beneficial effects of K2-7 in various diseases underscore the need for supplementation of K2-7 in the global diet as evidenced by clinical and molecular data. Some of the trials on vitamin K2-7 that enrolled people with chronic diseases such as diabetes were limited to 8–12 weeks duration. This underscores the lack of adequately-designed intervention trials for K2-7 that can truly capture the real-world evidence-based scenario in clinical therapeutics. Nevertheless, these trials, despite their limitations, provide a proof-of-concept for the utility of K2-7 intervention, which must necessarily be followed up with larger, better-designed and suitably longer duration trials.

AUTHOR CONTRIBUTIONS

SN conceived of the manuscript. DM and SN edited the manuscript. NJ, SA, PS, PG, AP, VB, YG, SP, and SJ wrote the manuscript and prepared the tables and figures under the supervision of SN. All authors read and approved the manuscript.

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GLOSSARY

AD Alzheimer's disease

AKT Protein kinase B/PKB

AMPK AMP-activated protein kinase

Bad Bcl-2-associated death promoter protein

Bax Bcl-2-associated X protein

Bcl-2 B-cell lymphoma 2

BMP Bone morphogenetic protein

CDK Cyclin-dependent kinases

CKD Chronic kidney disease

CVD Cardiovascular disease

ERK Extracellular signal-regulated kinase

Gas6 Growth arrest-specific protein 6

HCC Hepatocellular carcinoma

HepPPS Heptaprenyl pyrophosphatase synthetase

IKK-α Inhibitor of nuclear factor kappa-B kinase subunit alpha

JNK c-Jun N-terminal kinase

MAPK Mitogen-activated protein kinase

MGP Matrix Gla protein

MK Menaquinone

NF-κB Nuclear factor kappa light chain enhancer of activated B cells

OC Osteocalcin

OPG Osteoprotegerin

PARP Poly-ADP ribose polymerase

PI3K Phosphoinositide 3-kinase

PPAR-α Peroxisome proliferator-activated receptor

pRb Retinoblastoma protein

RANKL/RANK Receptor activator for nuclear factor kappa ligand/receptor

ROS Reactive oxygen species

SOCS Suppressor of cytokine signaling

STAT5B Signal transducer and activator of transcription 5B

TAG Triacylglycerol

TAK Transforming growth factor-β-activated kinase 1

TAM Tyro3, Axl, and Mer family

VKA Antagonist of vitamin K

VSMC Vascular smooth muscle cells

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In vitro assessment of the probiotic properties of an industrial preparation containing *Lacticaseibacillus paracasei* in the context of athlete health

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Intense physical activity is often associated with undesirable physiological changes, including increased inflammation, transient immunodepression, increased susceptibility to infections, altered intestinal barrier integrity, and increased oxidative stress. Several trials suggested that probiotics supplementation may have beneficial effects on sport-associated gastro-intestinal and immune disorders. Recently, in a placebo-controlled human trial, the AminoAlta™ probiotic formulation (AApf) was demonstrated to increase the absorption of amino acids from pea protein, suggesting that the administration of AApf could overcome the compositional limitations of plant proteins. In this study, human cell line models were used to assess *in vitro* the potential capacity of AApf to protect from the physiological damages that an intense physical activity may cause. The obtained results revealed that the bacteria in the AApf have the ability to adhere to differentiated Caco-2 epithelial cell layer. In addition, the AApf was shown to reduce the activation of NF-κB in Caco-2 cells under inflammatory stimulation. Notably, this anti-inflammatory activity was enhanced in the presence of partially hydrolyzed plant proteins. The AApf also triggered the expression of cytokines by the THP-1 macrophage model in a dose-dependent manner. In particular, the expression of cytokines IL-1β, IL-6, and TNF-α was higher than that of the regulatory cytokine IL-10, resembling a cytokine profile characteristic of M1 phenotype, which typically intervene in counteracting bacterial and viral infections. Finally, AApf was shown to reduce transepithelial permeability and increase superoxide dismutase activity in the Caco-2 cell model. In conclusion, this study suggests that the AApf may potentially provide a spectrum of benefits useful to dampen the gastro-intestinal and immune detrimental consequences of an intense physical activity.

KEYWORDS

aminoalta, CaCo-2, teer, superoxide dismutase, THP-1, NF-κB, L. paracasei DG, L. paracasei LPC-S01

Introduction

Both elite and recreational athletes commonly experience exercise-induced adverse health effects of too much exercise with the severity depending on the type and frequency of the physical activity. Strenuous exercise causes gastrointestinal symptoms in 30%–70% of athletes (Dimeo et al., 2004; Ter Steege et al., 2008; De Oliveira and Burini, 2009) and is associated with an increased risk of upper respiratory tract infections (URTIs) (Hull et al., 2012). Both adverse effects have been linked to an exercise-induced increase in intestinal permeability, altered immune function, and enhanced oxidative stress (Reid, 2001; Sadowska-Krepa et al., 2021). Several nutritional strategies have been proposed to treat or prevent the detrimental effects of physical activity, such as consuming beverages containing multiple transportable carbohydrates, limiting/avoiding fiber and solid foods hours/days before exercise, or supplementing the diet with vitamin C, D and E (Rowlands et al., 2012; Waterman and Kapur, 2012; De Oliveira et al., 2014; Cicchella et al., 2021), and antioxidants (Peake et al., 2007; Bowtell and Kelly, 2019). Supplementation with probiotics (Diaz-Jimenez et al., 2021; Tavakoly et al., 2021), "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014), plays an important role in maintaining normal physiology during exercise and to manage the adverse effects of those physically active. Reportedly, probiotics can positively influence human health principally by affecting the intestinal microbial ecosystem, regulating the activity of the gut-associated lymphoid tissue (GALT), and modulating the gene expression in the intestinal mucosa, so contributing to gut barrier preservation (i.e., reducing gut permeability), immune homeostasis promotion, and gut motility restoration (Dimidi et al., 2017; Ibrahim et al., 2020). These properties of probiotic microorganisms may explain the health benefits observed in athletes following probiotic supplementation, which include reduction of gastrointestinal symptoms in elite cyclists (Schreiber et al., 2021) and during a marathon race (Pugh et al., 2019), alleviation of URTI incidence after a marathon race (Tavares-Silva et al., 2021), amelioration of cardiorespiratory fitness in long-distance runners (Smarkusz-Zarzecka et al., 2020), promotion of favorable effects on self-reported muscle soreness and sleep quality in rugby players (Harnett et al., 2021), attenuation of circulating TNF-α in male baseball athletes (Townsend et al., 2018), and reduction of oxidative stress associated to exhaustive treadmill exercise in untrained subjects (Mooren et al., 2020). Moreover, recently, a probiotic formulation based on *Lacticaseibacillus paracasei* was shown to increase the maximum serum concentration of several amino acids when co-administered with plant protein (Jäger et al., 2020). The probiotic formulation used in this study, named AminoAlta™ (AApf), included 10 billion CFUs of two strains belonging to *Lacticaseibacillus paracasei*, which is one of the most commonly used bacterial species in commercial probiotic products. The species *L. paracasei* comprise numerous commercial strains including the world's first commercial probiotic, the Shirota strain, which was demonstrated to reduce infection incidence in athletes engaged in endurance-based physical activities (Gleeson et al., 2011) and to modulate the systemic and airways immune responses post-marathon (Vaisberg et al., 2019). Hundreds of other studies, also performed with the bacterial strains of the AApf, demonstrated that *L. paracasei* can provide a plethora of benefits in a strain-specific fashion for different health conditions and through different mechanisms ranging from the modification of the bacterial community structure of the intestinal microbiota, the modulation of intestinal short-chain fatty acid levels, the regulation of evacuation frequency, and the cross-talk with the intestinal mucosal immune system (Ferrario et al., 2014; Balzaretti et al., 2015; Cremon et al., 2018; Taverniti et al., 2021).

In this study, we assessed the hypothesis that the AApf, besides improving the absorption of amino acids from plant proteins, may also exert probiotic properties that can benefit athletes' health. To this aim, *in vitro* human cell line models have been implemented to measure the bacterial adhesion on enterocytes, the anti-inflammatory potential, the macrophage stimulatory activity, the epithelial permeability preservation, and the modulation of epithelial antioxidant capacity.

Materials and methods

Composition of the AminoAlta™ probiotic formulation

AminoAlta™ is a commercial probiotic product composed of 10 billion CFUs of *Lacticaseibacillus paracasei* cells [5 billion CFUs of strain *L. paracasei* LP-DG® (CNCM I-1572) and 5 billion CFUs of strain *L. paracasei* LPC-S01 (DSM 26760)] dispensed in a sachet containing 2.4 g of lyophilized product.

Bacterial adhesion to the Caco-2 cell layer

The adhesion of AApf to a Caco-2 cell layer was assessed as previously described (Guglielmetti et al., 2008) with few changes. In brief, Caco-2 cells were grown at 37°C in an atmosphere of 95% air and 5% carbon dioxide in Dulbecco's Modified Eagle's Medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine. For adhesion experiments, differentiated Caco-2 cells were used (i.e., 15 days after confluence). Approximately 2×10^8 bacterial cells were incubated with a monolayer of approximately 1×10^6 Caco-2 cells for 1 h at 37°C. Monolayers were washed three times with phosphate-buffered

saline pH 7.3 (PBS) to release unbound bacteria and incubated with 3 ml of methanol for 8 min at room temperature to fix cells. Afterwards, cells were stained with 3 ml of Giemsa stain solution (1:20; Carlo Erba, Milano, Italy) and left 30 min at room temperature in the dark. Finally, monolayers were washed three times with PBS, dried in an incubator for 1 h, and examined microscopically (magnification, ×400) under oil immersion. All experiments were performed in triplicate.

Study of NF-κB activation in Caco-2 cells

The activation of the nuclear factor κB (NF-κB) was studied by means of a recombinant Caco-2 cell line stably transfected with vector pNiFty2-Seap (InvivoGen, Labogen, Rho, Italy) as in Taverniti et al. (Taverniti et al., 2013). In brief, recombinant Caco-2 monolayers (approximately 5×10^5 cells/well), cultivated in the presence of 50 μg/ml zeocin, were washed with PBS and then incubated with 5×10^7 bacterial cells of the AApf suspended in fresh DMEM containing 100 mM HEPES (pH 7.4), resulting in a "multiplicity of infection" (MOI) of approximately 100. In a different set of experiments, Caco-2 monolayers were also co-incubated with six different commercial supplements of partially hydrolyzed vegetal proteins (PHVP; 10 μg/ml final concentration) resuspended in PBS. The tested commercial supplements were as follows: NUTRALYS pea protein (consisting of pea proteins; code PHVP-A), Organic protein™ (pea, brown rice and chia seed proteins; PHVP-B), KOS® (pea, coconut milk and flax seed proteins; PHVP-C), Sunwarrior® (pea and hemp proteins; PHVP-D), Vega protein (pea and brown rice proteins; PHVP-E), Raw Organic Protein-Garden of life (pea and brown rice proteins; PHVP-F). Pro-inflammatory stimulation of Caco-2 cells was carried out by adding 2 ng/ml of interleukin (IL)-1β. After incubation at 37°C for 4 h, the activity of the secreted embryonic alkaline phosphatase (SEAP) reporter enzyme was quantified in the supernatant using the Quanti-Blue reagent (Invivogen) according to the manufacturer's protocol using a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA) at 655 nm OD. Three independent experiments were conducted in duplicate for each condition.

THP-1 human macrophage cell line activation assessment: Cell culture, growth conditions, and stimulation protocol

The growth medium for THP-1 cells consisted of RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Milan. Italy), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich Pty Ltd., Darmstadt, Germany). Cells were seeded at a density of 1×10^6 cells/well in 12-well plates and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differentiation was induced by treating cells with 20 ng/ml of phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) for 24 h. Afterwards, cells were washed once with sterile PBS to remove non-adherent cells. One hour before the bacteria were added to the cells, the culture medium was replaced with RPMI 1640 medium without FBS to allow the cells to adapt. Finally, differentiated THP-1 cells were stimulated with bacteria at MOIs 10 and 50 for 4 h.

Preparation of RNA and real-time quantitative reverse transcription PCR (qRT-PCR)

After incubating THP-1 cells at 37°C for 4 h, the supernatant was carefully removed from each well and the total cellular RNA was isolated from adhered THP-1 cells with the RNeasy Plus Mini Kit—Qiagen (Qiagen). Afterwards, RNA concentration and purity was determined by spectrophotometric analysis (Multiskan SkyHigh, Thermo Fisher Scientific) and through electrophoresis on 0.8% agarose gel stained with GelRed Nucleic Acid Staining (Millipore). Total mRNA reverse transcription to cDNA was performed with the RNeasy Mini Kit (Qiagen) with 1 μg of RNA, using the following thermal cycle: 2 min at 42°C for DNase activity, then 15 min at 42°C, and 3 min at 95°C, as per the manufacturer's instructions. qRT-PCR was carried out to measure the mRNA expression levels of cytokine genes using the SsoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the manufacturer's instructions. The primers used were as follows (5′-3′): IL-10 forward AGC AGAGTGAAGACTTTCTTTC; IL-10 reverse CATCTCAGA CAAGGCTTGG; TNF-α forward TCAGCTCCACGCCATT; TNF-α reverse CCCAGGCAGTCAGATCAT; IL-1β forward TGGCAATGAGGATGACTTGTTC; IL-1β reverse CTGTAG TGGTGGTCGGAGATT; IL-6 forward CGGTACATCCTC GACGGCAT; IL-6 reverse TCACCAGGCAAGTCTCCTCAT. All primers were designed previously, and their specificity was assessed with melting curves during amplification and by 1% agarose gels (Taverniti et al., 2012). Quantitative PCR was carried out according to the following thermal cycle: initial hold at 95°C for 30 s and then 41 cycles at 95°C for 10 s and 60°C for 5 s. Gene expression was normalized to the reference genes β-actin (ACTB) and Ribosomal Protein L37a (RPL37A). The primers used were as follows (5′-3′): ACTB forward ATTGCCGACAGG ATGCAGAA; ACTB reverse GCTGATCCACATCTGCTG GAA; RPL37A forward ATTGAAATCAGCCAGCACGC; RPL37A reverse AGGAACCACAGTGCCAGATCC. The amount of template cDNA used for each sample was 25 ng. All results regarding cytokine mRNA expression levels are reported as the fold of induction (FOI) respective to the

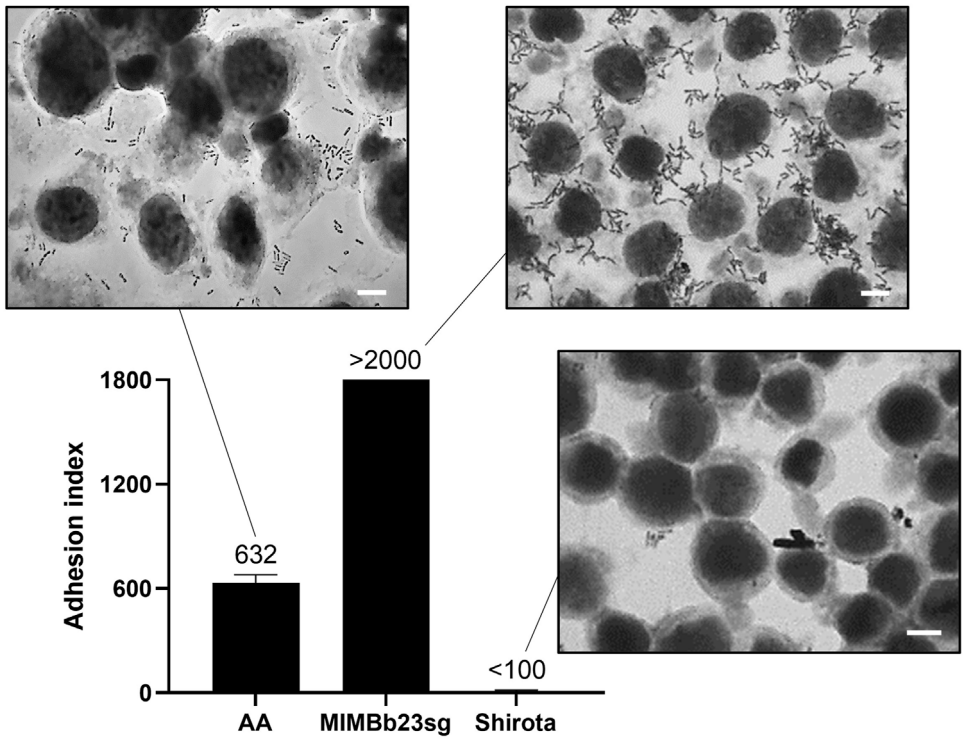

FIGURE 1
Adhesion of the bacterial cells within the AminoAlta™ probiotic formulation to a Caco-2 cell monolayer as observed with Giemsa staining under a light microscope. Adhesion quantification is reported as adhesion index (i.e., bacterial cells adhered to 100 Caco-2 cells). Histograms represent means of at least three independent experiments conducted in duplicate. The vertical bars indicate standard deviations. AA, AminoAlta™ formulation; MIMBb23sg, *Bifidobacterium bifidum* MIMBb23sg (positive control); Shirota, *Lacticaseibacillus paracasei* Shirota (negative control). White bar, 10 µm.

control (namely unstimulated THP-1), to which we attributed a FOI of 1.

Assessment of the transepithelial electrical resistance (TEER) in Caco-2

The effect of the probiotic formulation on epithelial integrity was assessed with a Caco-2 cell layer by transepithelial electrical resistance (TEER) measured after 4 and 24 h. For the analysis, a fully differentiated Caco-2 cell layer obtained by growing cells in 6 well plate transwell inserts for 21 days was used. The apical side of the Caco-2 cell layers was incubated with the AApf resuspended in DMEM medium without antibiotics at a MOI of 100. TEER was measured using an epithelial voltohmeter (Milicell ERS-2 Voltohmmeter; World Precision Instruments, Hitchen, UK). This instrument uses a pair of electrodes ("chopsticks"): one electrode was placed inside the basolateral culture medium, and the shorter electrode was placed in the transwell insert within the apical culture medium. Cells were never in contact with the electrodes. Instrument calibration and the experiment were carried out according to the manufacturer's instructions.

Assessment of the cellular antioxidant activity in Caco-2 cells

The cellular antioxidant activity (CAA) of AminoAlta™ was evaluated with the Caco-2 cell model by measuring the relative amount of intracellular reactive oxygen species (ROS) as describe in Wolfe and Liu (Wolfe and Liu, 2007) and Xing et al. (Xing et al., 2015) with few modifications. In brief, Caco-2 cells were seeded at a density of 1×10^4 cells/well on a black 96-well microplate with clear bottom in 100 µl DMEM complete medium for 72 h at 37°C to reach 80% confluence. Then, Caco-2 cells were treated with 100 µl of 10 µM 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich) up to 30 min at 37°C. Subsequently, the cells were washed with PBS and treated with 100 µl of different concentration of 2,2′-azobis (2-methylpropionamidine) dihydrochloride (ABAP; from 0.2 to 2 mM), together with

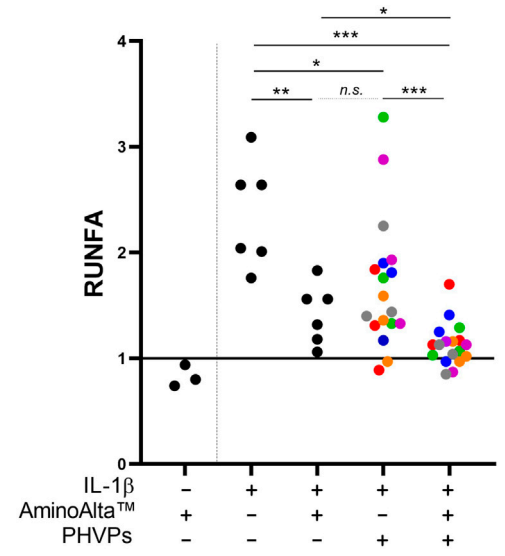

FIGURE 2
Study of the activation of the NF-κB transcriptional regulator in a Caco-2 cell layer transfected with an alkaline phosphatase (SEAP) reporter vector. Measurements of the SEAP activity were carried out after incubation of the Caco-2 cell layer without or with stimulation with IL-1β (2 ng/ml), the AminoAlta™ probiotic formulation, and different partially hydrolyzed vegetal protein commercial preparations (PHVPs). RUNFA, relative units of NF-κB activation, calculated as normalized SEAP activity. Asterisks indicate statistically significant differences according to a two-tailed unpaired Student's t-test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; n. s., not significant.

suspensions of AApf cells dissolved in Hanks' balanced salt solution at multiplicity of infections (MOI) of 50, 100, 200, and 1,000. N-acetyl cysteine was used as positive control (not shown). Fluorescence was measured with Fluoroskan Ascent FL (Thermo Fisher Scientific) for 6 cycles at 5-min intervals (λ excitation = 485 nm and λ emission = 538 nm). Fluorescence measurements of Caco-2 cells treated with DCFH-DA were used as blank, whereas measurements of cells treated with DCFH-DA and ABAP (without the treatment with AApf) were used as the control. Blank was subtracted from fluorescence measurements. For CAA assessment, the higher fluorescence emissions (expressed as relative fluorescence units, RFUs) were compered between samples (i.e., AApf-treated) and controls.

Statistical analysis

Statistical calculations were performed using the software program GraphPad Prism 5. A two-tailed unpaired Student's t-test was used to find the significant difference between two groups. Before *t*-test, one-way analysis of variance (ANOVA) was performed for data with more than two groups. *p* value <0.05 was considered for statistical significance. All the observed significant differences had $p < 0.05$ also with a non-parametric test (Mann-Whitney U test; not shown).

Results

The bacteria in the AminoAlta™ probiotic formulation (AApf) possess the ability to adhere on Caco-2 enterocyte-like cells

The differentiated Caco-2 epithelial cell layer was used to test the potential ability of the bacterial cells within the AApf to adhere on human enterocytes. The experiment was carried out including two control strains, *Bifidobacterium bifidum* MIMBb23sg and *Lacticaseibacillus paracasei* Shirota, which have been previously demonstrated to be strongly adhesive (Guglielmetti et al., 2009) and non-adhesive (Botes et al., 2008; Balzaretti et al., 2015), respectively. The reference bacteria performed as expected, with an adhesion index (i.e., bacterial cells per 100 Caco-2 cells) higher than 2000 for the *B. bifidum* strain and lower than 100 for the Shirota strain (Figure 1). Furthermore, also the bacterial cells within the AApf displayed an adhesive phenotype, corresponding to an adhesion index of about 600 (Figure 1).

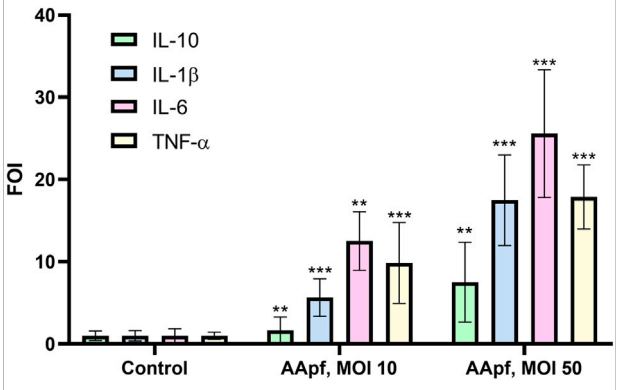

FIGURE 3
Gene expression analysis by qRT-PCR in THP-1 human macrophages unstimulated (Control) and after 4 h of stimulation with the AminoAlta™ probiotic formulation (AApf) at a multiplicity of infection (MOI) of 10 and 50. Expression levels of IL-10, IL-1β, IL-6, and TNF-α are shown as the fold change in induction (FOI) relative to expression by the control (unstimulated macrophages), which was set at a value of 1. Data are means of results from three (control and MOI 10) or two (MOI 50) independent experiments ± standard deviations. Asterisks indicate statistically significant differences (according to a two-tailed unpaired Student's t-test) from results for unstimulated THP-1 cells. ***, $p < 0.001$; **, $p < 0.01$.

AApf prevents NF-κB activation in Caco-2 epithelial cells under inflammatory stimulation

The anti-inflammatory activity of AApf was assessed using the Caco-2/NF-κB reporter system. The experiments were carried out by stimulating for 4 h the recombinant Caco-2 cell layer at baseline and in presence of a pro-inflammatory stimulation with IL-1β. At baseline, AApf only marginally affected NF-κB activation [relative units of NF-κB activation (RUNFA) of 0.8 ± 0.1, as mean ± standard deviation] (Figure 2). The addition of IL-1β approximately doubled the activation levels of NF-κB (RUNFA = 2.4 ± 0.5). Notably, the presence of AApf significantly reduced NF-κB activation to levels close to those at baseline (RUNFA = 1.4 ± 0.3) (Figure 2). Subsequently, the same test was carried out incubating the recombinant Caco-2 cell layer in the presence of six different commercial preparations of partially hydrolyzed vegetal proteins (PHVPs) together with the inflammatory stimulus IL-1β. NF-κB activation was slightly but significantly reduced by the PHVP formulations (RUNFA = 1.7 ± 0.6). Notably, the reduction was greater when AApf was added (RUNFA = 1.1 ± 0.2), suggesting an additive or synergistic action between AApf and PHVPs (Figure 2).

AApf triggers the expression of cytokines by THP-1 cells

We used the THP-1 cell line as a simplified human macrophage model (Tedesco et al., 2018) to test the immunostimulatory properties of AApf at two different concentrations of bacterial cells: MOI 10 and 50. To this aim, we quantified by RT-qPCR the gene expression of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-10. Both AApf concentrations triggered the expression of cytokines in a dose-dependent manner (Figure 3). In particular, the AApf stimulus increased the expression of the cytokines IL-1β (FOI 5.7 ± 2.3 at MOI 10 and 17.5 ± 5.5 at MOI 50), IL-6 (FOI 12.5 ± 3.6 at MOI 10 and 25.6 ± 7.8 at MOI 50), and TNF-α (FOI 9.9 ± 4.9 at MOI 10 and 17.9 ± 3.9 at MOI 50) more than that of the regulatory/anti-inflammatory IL-10 (FOI 1.7 ± 1.6 at MOI 10 and 7.5 ± 4.9 at MOI 50) (Figure 3), suggesting that THP-1 macrophages stimulated with AApf are more prone to express markers that characterize a M1 phenotype (IL-1ß, TN-α, and IL-6) rather than a M2 phenotype (IL-10) (Chanput et al., 2013).

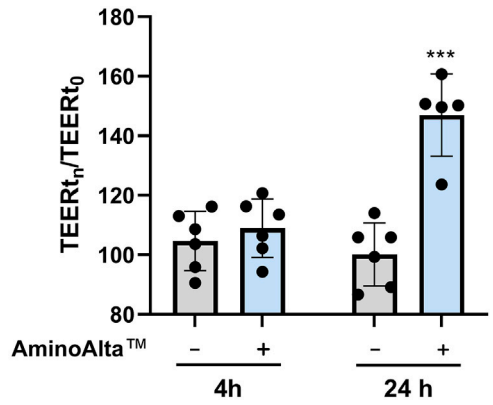

FIGURE 4
Transepithelial electrical resistance (TEER) measurements of Caco-2 cell layers exposed to the AminoAlta™ probiotic formulation (+) or unstimulated (–) after 4 and 24 h. Data are means of results from six independent experiments ± standard deviations. Asterisks indicate statistically significant differences according to a two-tailed unpaired Student's t-test. ***, $p < 0.001$.

AApf preserves the transepithelial permeability of the Caco-2 epithelial cell layer

The ability of AApf to influence transepithelial permeability was assessed on Caco-2 monolayer as changes in the transepithelial electrical resistance (TEER) values at two incubation time points (4 and 24 h). To calculate the relative changes in TEER, the measurement was immediately performed after the stimulation of Caco-2 with AApf ($TEERt_0$). Cell monolayers without bacterial incubation were considered as a control group. The obtained results showed that AApf, employed at the same bacterial cell concentration adopted for the NF-κB activation test, can significantly enhance the TEER of the Caco-2 layer after 24 h of incubation (Figure 4), suggesting its potential ability to ameliorate the epithelial barrier function.

AApf exerts an antioxidative activity in Caco-2 cells

The CAA assay was used to reveal the total antioxidative capacity of the probiotic formulation under study by measuring ROS accumulation in Caco-2 cells. The experiment was carried out damaging Caco-2 cells with different concentrations of ABAP, and testing AApf at 4 different MOIs. The obtained results showed a dose-dependent ability of AApf to lower ROS in Caco-2 cells, with a significant reduction already observed at the lowest bacterial cell concentration tested (MOI = 50) (Figure 5).

Discussion

Increasing attention is given to the use of probiotic supplements to protect from the undesirable physiological changes that may be induced by strenuous physical activity. According to the available scientific literature, the effectiveness of

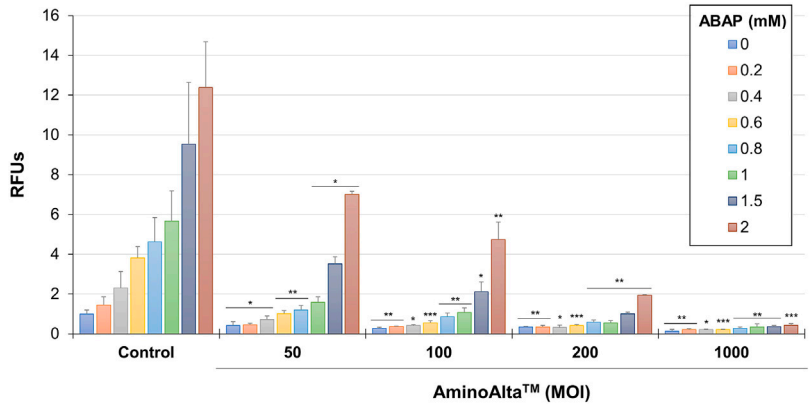

FIGURE 5
Cellular antioxidant activity of AminoAlta™ assessed in Caco-2 cells damaged by different concentrations of 2,2′-azobis (2-methylpropionamidine) dihydrochloride (ABAP). Control, Caco-2 cells without AminoAlta™ treatment. RFUs, relative fluorescence units. MOI, multiplicity of infection. Asterisks indicate statistically significant difference compared to the corresponding control as determined through a two-tailed unpaired Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

probiotic supplementation in athletes can is associated with improved gut-barrier-function, the attenuation of inflammatory responses, and the improvement of the epithelial antioxidant status after exhaustive exercise (Marttinen et al., 2020; Diaz-Jimenez et al., 2021). Thus, in a recent consensus document, the International Society of Sports Nutrition (ISSN) stated that certain probiotics may reduce URTIs and improve the integrity of the gut-barrier function in athletes (Jäger et al., 2019). Nonetheless, the ISSN also highlighted the importance to assess the potential properties of probiotics in trials carried out for any specific formulation, since probiotic health-promoting capabilities are strain-specific, particularly regarding immunomodulation (Hill et al., 2014); and depend on dose, delivery form, and method of administration (Jäger et al., 2019). In addition, any aspect of a probiotic formulation, including excipients and the protocol adopted in the industrial manufacturing process, may influence the properties of a probiotic product (Fiore et al., 2020). It is, in fact, demonstrated for a few well-known commercial probiotic strains that expression and presence of probiotic niche factors and effector molecules may be affected during industrial production (Duboux et al., 2021). For these reasons, in our study, we did not test microbial cells prepared in small laboratory scale (as often done in other studies), but we employed the industrial bacteria within the final commercial probiotic product.

The ability to adhere on the intestinal epithelium is conventionally considered a primary prerequisite of probiotic microorganisms, which can favor the transient colonization, pathogens exclusion, and the crosstalk with the intestinal mucosa. Thus, we first tested the ability of the bacterial cells within the AApf to adhere on the differentiated Caco-2 cell layer, which is a model for the intestinal epithelial barrier (Sambuy et al., 2005) commonly used to test the adhesion ability of probiotics (Guglielmetti et al., 2008; Guglielmetti et al., 2009). The obtained results demonstrated that the AminoAlta™ bacteria possess a significant adhesion ability, which is a feature reported to be strain-specific among members of the *Lacticaseibacillus paracasei* species (Balzaretti et al., 2015; Fonseca et al., 2021).

Subsequently, we tested the immunomodulatory properties of the AminoAlta™ formulation by assessing in epithelial Caco-2 cells the ability to reduce the activation of NF-κB, a transcriptional factor regulating the expression of proinflammatory cytokines (Baldwin, 1996) that is exploited as a therapeutic target in human inflammatory diseases (Yamamoto and Gaynor, 2001). Intense long physical exercise induces inflammation systemically and in the gut of athletes (Van Wijck et al., 2011). In our model, we used IL-1β to mimic a pro-inflammatory stimulus. The resulting increase in NF-κB activation was significantly mitigated by the presence of the AApf. This result confirms previous findings that showed that the *L. paracasei* bacteria contained in the AApf can reduce NF-κB activation in the same *in vitro* cell line under inflammatory stimulation (Balzaretti et al., 2015). Here, we additionally demonstrated that AminoAlta™ bacteria exert anti-inflammatory activity also when employed as industrial lyophilized biomass within the 4 h of experiment time.

The anti-inflammatory properties of *L. paracasei* were proposed to be connected to an unidentified peptide factor produced by the bacterium during the fermentation process (Zagato et al., 2014; Balzaretti et al., 2015), which can potentially derive from the hydrolysis of the proteins/polypeptides present in the culture medium. Interestingly, we

found an increase of the anti-inflammatory effect when the AApf was used in the experiment together with commercial preparations of partially hydrolyzed plant proteins (PHVPs). Animal and vegetable protein hydrolysates have been shown to exert anti-inflammatory properties in several studies (Kiewiet et al., 2018; Kim et al., 2021; Montserrat-De La Paz et al., 2021). Also in our experiments, the different PHVPs exerted a mild but significant reduction of NF-κB activation in Caco-2 cells, which was significantly enhanced by the presence of the AApf. The AminoAlta™ bacteria were shown in a previous publication to be able to hydrolyze plant (rice and pea) proteins (Jäger et al., 2020). Therefore, we can hypothesize that the bacteria in the AApf carried out a partial additional hydrolysis of the vegetal proteins during the 4 h of co-incubation in contact with Caco-2 cells, releasing peptides that may potentially exert anti-inflammatory activities. Reportedly, the ability of lactic acid bacteria to generate anti-inflammatory peptides from the hydrolysis of food proteins has been already reported for instance for casein (Stuknyte et al., 2011) and cereal proteins (Galli et al., 2018).

Intense and prolonged (arduous) exercise is reported to provoke immunity suppression and increased susceptibility to infections (Simpson et al., 2020). Strenuous physical activity, in fact, has the potential to alter transiently (i.e., from hours to days) immune functionality, including NK cell activity, and antigen presentation and cytokine production by monocytes/macrophages (Woods et al., 2000; Nieman and Wentz, 2019). In our study, the AApf was shown to trigger cytokine gene transcription in macrophages *in vitro*. Macrophages are immune cells that sense the molecules and stimuli present in the microenvironment and polarize towards a specific phenotype depending on the surrounding conditions, with a high level of plasticity between the M1 and M2 phenotypes that, once activated, can permit the resolution of inflammation (Liu et al., 2020). Specifically, the observed increased expression of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α evokes a pattern of markers typical of the M1 phenotype. This population of activated macrophages is associated to increased phagocytic capacity, tumor regression and defense against bacterial and viral infection (Ley, 2017). In particular, M1 polarized macrophages are the first line of defense against intracellular pathogens through mechanisms of endocytosis, production of reactive oxygen species (ROS), increase of antigen-presenting ability, and also by the induction of a Th1 switch on CD4 T cells, which in turn potentiate the response against infections (Atri et al., 2018).

Our results show that AApf may exert both anti-inflammatory (reduced activation of NF-κB) and pro-inflammatory (enhanced expression of cytokines) activities. However, these results are not in contradiction, since they were obtained in different cell models. The NF-κB activation was assessed in Caco-2 cells, i.e. an enterocyte model, whereas the induction of pro-inflammatory cytokines in THP-1 cells, which are monocytes differentiated to macrophages. The intestinal epithelial cells are continuously exposed to different microbial cells and, for this reason, they trained to mainly develop tolerogenic responses. On the contrary, macrophages are antigen presenting cells that work as sentinels of the immune system, in charge of patrolling the environment, being more prone to rapidly mount inflammatory responses upon the (first) encounter with microbial cells. The different immunomodulatory attitude in the cross-talk towards epithelial or proper immune cells have previously been reported for probiotic bacteria. For instance, *Lacticaseibacillus paracasei* strain DG displayed an anti-inflammatory phenotype on epithelial cells, by reducing NF-κB in Caco-2 model (Balzaretti et al., 2015), whereas showed an immunostimulatory phenotype when used to stimulate THP-1 cells, by inducing IL-8, TNF-α and CCL20 (Balzaretti et al., 2017). Analogously *Lactobacillus helveticus* MIMLh5 was shown to strongly reduce NF-κB activation in Caco-2 cell layer, while elicited a Th1 response in U937 macrophage cell line and in macrophages isolated from mouse bone marrow (Taverniti et al., 2013).

Strenuous exercise was also associated with the disruption of the intestinal barrier integrity, resulting in increased gut permeability (Janssenduijghuijsen et al., 2016; Ribeiro et al., 2021), causing local and systemic low-grade chronic inflammation, which is mechanistically linked with several pathological conditions ranging from inflammatory bowel diseases and metabolic syndrome to food allergy and celiac disease (Bischoff et al., 2014). Probiotic administration has been often proposed as a useful tool to improve intestinal barrier function, nonetheless convincing experimental data are still missing, and a wide variation exists among different microbial strains (Ramos et al., 2013; Bron et al., 2017). Reportedly, the probiotic mixture VSL#3 was shown to protect the epithelia barrier by preserving tight junction protein expression in a mouse model of colitis (Mennigen et al., 2009) and in a rat model of alcoholic intestinal injury (Chang et al., 2013). In a different study, the probiotic strain *Escherichia coli* Nissle 1917 was shown to up-regulate the expression of the junction-associated protein zonula occludens 1 (ZO-1) in the intestinal epithelial cells of DSS-treated mice (Ukena et al., 2007). Furthermore, some strains of *Lactiplatibacillus plantarum* were shown to enhance intestinal barrier function according to *in vitro* assays based on transepithelial electrical resistance (TEER) measurement (Anderson et al., 2010a) and tight junction genes expression analysis (Anderson et al., 2010b) in Caco-2 epithelial cell layers. Notably, the administration of the probiotic strain *L. paracasei* DG, which is included in the AApf, was recently shown *in vivo* to reduce in mouse colonic mucosa the expression of zonulin (Taverniti et al., 2021), an enzyme that regulates intestinal barrier function promoting intestinal permeability (Fasano, 2011). In our study, the AApf was shown to significantly increase the barrier function in Caco-2 cell layer after 24-h

co-incubation, suggesting its potential capability of preventing or reducing intestinal permeability. The mechanisms behind the effects of the AApf or other probiotics on the intestinal epithelial permeability are not precisely known, but it was proposed that the probiotic ability to reduce inflammation at epithelial level can provide a substantial contribution. This hypothesis can be applied in our study, considering our data that showed the ability of the AApf to prevent the activation of the pro-inflammatory transcriptional regulator NF-κB in the Caco-2 intestinal epithelial cell model.

High intensity physical activity increases the production of ROS, most of which are generated in the form of radical superoxide ($O_2^{\bullet -}$). Experimental evidence showed that ROS, which are associated with various gastrointestinal inflammatory and metabolic disorders (Bhattacharyya et al., 2014), can be detoxified with the contribution of probiotic microorganisms (Wang et al., 2017b). Reportedly, in fact, probiotics can exert significant antioxidant abilities (Lin and Yen, 1999; Persichetti et al., 2014; Wang et al., 2017a). For instance, a high antioxidative effect was reported for *Lacticaseibacillus rhamnosus* and *Lactiplantibacillus plantarum* in ABAP-damaged HepG2 and Caco-2 cell lines, respectively (Wolfe and Liu, 2007; Mu et al., 2019). We found a similar result with the AApf, which significantly reduced ROS accumulation in the Caco-2 cell layer in a dose-dependent fashion also in the presence of different concentrations of the peroxyl radical generator ABAP. Zhang et al. reported that the ability of *Lactiplantibacillus plantarum* C88 to protect Caco-2 cells against oxidative injury was determined by an exopolysaccharide (EPS), which was shown to raise the SOD activity in a dose-dependent manner (Zhang et al., 2013). Interestingly, strain *L. paracasei* DG, which is contained in the AApf, secrete a rhamnose-rich hetero-EPS that covers the outer cell surface (Balzaretti et al., 2017). We can therefore speculate that, similarly to strain C88, the EPS of strain DG could contribute to the antioxidant properties of AApf.

In the intestinal mucosa, oxidative stress and inflammation are interconnected, and contribute to epithelial barrier damage (Chen et al., 2021). Potentially, the AApf ability of reducing ROS in enterocytes, together with the observed anti-inflammatory activity and epithelial barriers preservation, could contribute to protect the integrity of the intestinal mucosa under the stress conditions generated by the strenuous physical activity. Inflammation and oxidative stress are the underlying mechanism of exercise-induced muscle damage, resulting in reduced muscle strength, range of motion and increased muscle soreness. The extent of the muscle damage depends on the intensity and duration of the exercise. While the exercise-induced inflammatory response is crucial to muscle repair and regeneration, nutritional strategies to manage inflammation and oxidative stress are crucial for optimal recovery. Faster recovery allows athletes to train harder and more frequently and results in faster training adaptations and increased performance (Jäger et al., 2016).

This study has several limitations, primarily consisting in the fact that only *in vitro* experiments have been performed, and that the conditions in the gut during physical exercise were only marginally mimicked in our tests. Nonetheless, we used *in vitro* models that are widely adopted in scientific studies and have been demonstrated to be good predictors of the *in vivo* activity. Furthermore, in our opinion, the possibility that the results of this study may effectively prognosticate an effect on human health *in vivo* is favored by two additional facts: 1) we used in the experiments the same industrial formulation available on the market, which has been already employed in a clinical trial (Jäger et al., 2020), and 2) the two bacterial strains in AApf have been already demonstrated *in vivo* to survive the gastrointestinal transit (Balzaretti et al., 2015; Arioli et al., 2018; Radicioni et al., 2019; Koirala et al., 2020).

Conclusion

This study suggests that the AminoAlta™ probiotic formulation may provide a range of benefits to counteract the gastro-intestinal and immune detrimental consequences of intense physical activity. Specifically, our study suggests that the AApf may exert the activities that are considered the mechanistic bases for the beneficial effects observed in athletes upon probiotic supplementation, such as the prevention of inflammation at the epithelial level, the stimulation of macrophage activity, the preservation of epithelial barrier integrity, and the enhancement of endogenous epithelial oxidant scavenging capacity.

Although derived from *in vitro* experiments, the results of this study provide the rationale for further investigations that will be based on clinical trials aimed to confirm the health-promoting properties of the AminoAlta™ probiotic product in an athletic population.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

SG, VV, and WF contributed to conception and design of the study. LB, VV, RF, and MM performed the laboratory experiments. SG wrote the first draft of the manuscript. LB, VV, RJ, VT, and SG wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

Authors LB, VV, RF, MM, and FW were employed by Sofar S.p.A., the company that commercialized the probiotic supplement investigated. RJ was employed by Increnovo LCC and a consultant to Sofar S.p.A. SG is a consultant to Sofar S.p.A. Sofar S.p.A. had a role in study design, data collection, and decision to publish.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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