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REVIEWS: CURRENT TOPICS

Probiotics as an emerging therapeutic strategy to treat NAFLD: focus on molecular and biochemical mechanisms

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is currently the most common liver disease worldwide, both in adults and in children. NAFLD is characterized by aberrant lipid storage in hepatocytes (hepatic steatosis) and inflammatory progression to nonalcoholic steatohepatitis. Evidences so far suggest that intrahepatic lipid accumulation does not always derive from obesity. Gut microbiota has been considered as a regulator of energy homeostasis and ectopic fat deposition, suggesting its implications in metabolic diseases. Probiotics are live microbial that alter the enteric microflora and have beneficial effects on human health. Although the molecular mechanisms of probiotics have not been completely elucidated yet, many of their effects have proved to be beneficial in NAFLD, including the modulation of the intestinal microbiota, an antibacterial substance production, an improved epithelial barrier function and a reduced intestinal inflammation. Given the close anatomical and functional correlation between the bowel and the liver, and the immunoregulatory effects elicited by probiotics, the aim of this review is to summarize today's knowledge about probiotics in NAFLD, focusing in particular on their molecular and biochemical mechanisms, as well as highlighting their efficacy as an emerging therapeutic strategy to treat this condition.

Keywords: Probiotic; NAFLD; Insulin resistance; Inflammation; Therapy

1. Introduction

Intestinal microflora has been first claimed to have a beneficial influence on human health over a century ago, and the ensuing research has by now soundly confirmed this concept. In the gut live about 10^{14} bacterial cells, including up to 2000 species dominated by anaerobic bacteria [1]. Intestinal microflora benefits from a constant nutrient flow, a stable temperature and appropriate niches for various metabolic requirements provided by the intestinal environment. Likewise, the host benefits from the ability of the intestinal microflora to synthesize vitamin K, exert trophic effects on intestinal epithelial cells, salvage energy from unabsorbed food by producing short-chain fatty acids (SCFA), inhibit the growth of pathogens, sustain intestinal barrier integrity, maintain mucosal immune homeostasis and participate to the xenobiotic metabolism system [2,3]. Probiotics are live microbes able to modulate the intestinal microflora and enhance body health. At birth, the gastrointestinal tract is a sterile environment. Within a few months after birth, a relatively stable microbial population is established [3,4]. This abundant, diverse and dynamic intestinal microflora normally lives in a complex, symbiotic relationship with the eukaryotic cells of the mucosa. *Firmicutes* are the most representative bacteria among phyla found in the human colon, and include *Clostridia* and lactic acid bacteria (LAB), and *Bacteroidetes* [3,5]. However, several factors, such as age, diet, hygienic habit, infection and antibiotic therapy, can modify the microbiota composition. Recently, gut microbiota has been considered as a regulator of energy homeostasis and ectopic fat deposition, evidencing its implications in metabolic diseases [6,7]. In particular, obese people were shown to have lower *Bacteroidetes* and more *Firmicutes* in their distal gut compared to lean control, and this alteration was abolished after diet-induced weight loss [8]. Moreover, high-fat-fed animals present gut microbiome with an increased number of transport proteins and enzymes involved in absorption and fermentation of simple sugars and host glycans. In return, these substances can be more utilized for hepatic lipogenesis by increasing the capacity of hosts to harvest energy from their diet [9]. Moreover, in healthy subjects, the microbiote suppresses the expression of a fasting-induced adipocyte factor (*Fiaf*, also known as angiopoietin-like protein 4), a lipoprotein lipase inhibitor, which is produced not only by the intestine, but also by liver and adipose tissue, and thereby being an important regulator of peripheral fat storage [10].

The majority of patients with nonalcoholic fatty liver disease (NAFLD) are either overweight or obese, and there is convincing evidence that NAFLD is a component of the metabolic syndrome [11]. NAFLD is currently the most common liver disease worldwide, both in

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adults and in children. It is characterized by an aberrant lipid storage in hepatocytes (hepatic steatosis) and an inflammatory progression to nonalcoholic steatohepatitis (NASH). Pathologically, there are several patterns of disease that resemble the alcoholic liver disease, but the sine qua non condition for NAFLD recognition is the macrovesicular steatosis or fatty liver. Simple steatosis remains a benign process in most affected people and seems to be well tolerated [12,13]. However, some patients develop superimposed necroinflammatory activity with a nonspecific inflammatory infiltrate and hepatocyte ballooning with Mallory's hyaline, which are the driving force for the development of fibrosis, as observed in NASH [14]. Likely, a minority of these patients develop cirrhosis, which may become complicated by hepatocellular carcinoma. Probiotics have been proposed in the treatment and prevention of many conditions. The mechanisms of these effects are multiple, the vast majority being related to the regulation of the immune system. Given the close anatomical and functional correlation between the bowel and the liver, and the immunoregulatory effects elicited by probiotics, the aim of this review is to summarize the probiotics research in NAFLD, specifically focusing on their molecular and biochemical mechanisms and highlighting their efficacy as an emerging therapeutic strategy to treat this condition.

2. Gut–liver axis

Due to its anatomical links to the gut, the liver is a major filter organ and a first-line defense for the host. The liver is constantly exposed to gut-derived bacterial fractions or metabolites, and it is an important site for bacterial phagocytosis and clearance, as it hosts more then 80% of the body's macrophages. In particular, Kupffer cells, the resident macrophages of the liver, effectively limit the amount of endotoxin and phagocyte bacteria carried through the portal vein, thus playing a pivotal role in the clearance of systemic bacterial infections [15]. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) to detect the presence of pathogens. Even low amounts of PAMPs, such as lipopolysaccharide (LPS), lipopeptides, unmethylated DNA and double-stranded RNA, evoke intense inflammatory reactions.

Considering that the gut hosts more than 99% of the bacterial mass in the body, intestinal microbiota is the principal source of bacterial-derived PAMPs both in health and disease. In addition to their role in innate immunity, TLRs also play a major role in the regulation of inflammation. Several TRL endogenous ligands, termed *damage-associated molecular patterns*, act as a signal of the presence of necrosis and subsequent trigger of inflammation [16–18].

The healthy liver contains low mRNA levels of TLRs (TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10) and signaling molecules (i.e., CD14, MD-2 and MyD88) as compared with other organs, suggesting that the low expression of TLR signaling molecules may contribute to the high tolerance of the liver to TLR ligands deriving from the intestinal microbiota [19,20].

In chronic liver diseases, for instance, cirrhosis, structural changes of the intestinal mucosa (e.g., loss of tight junctions, widening of intercellular spaces, vascular congestion, defects in the mucosal immune system) promote the loss of the barrier function and allow for translocation of bacteria and bacterial PAMPs [20]. Many pro-inflammatory effects of PAMPs are a consequence of TLR-induced secretion of inflammatory mediators, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, as demonstrated in vitro and in vivo [21].

The gut–liver axis is indicative of a tight linkage between the health of the intestinal tract and that of the liver. In fact, there is growing evidence of how alteration of the gut microflora dysbiosis may affect liver pathology. An altered intestinal bacterial flora because of stress or wrong nutritional habits could play an important role in the pathogenesis or the development of NAFLD. On the basis that a shift in the gut microbiota enteric profile, due to bacterial overgrowth, may contribute to the pathogenesis of NAFLD, treatments able to manipulate enteric flora, such as probiotics or prebiotics, have been proposed.

Normally, intestinal anaerobic bacteria outnumber aerobic bacteria, the latter being responsible for bacterial translocation. Thus, anaerobic bacteria, suppressing the colonization and growth of potentially invasive microbes, exert an important role in maintaining gastrointestinal health and in reducing the translocation of potentially dangerous microbes. Conversely, selective elimination of anaerobic bacteria promotes intestinal bacterial overgrowth and translocation. Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, enterococci and streptococci not only represent the species that are most proficient at translocation but also cause the large majority of infections in patients with cirrhosis [22].

3. Key features of NAFLD: insulin resistance and inflammation

Insulin resistance (IR) plays a crucial pathophysiological role in the development and progression of NAFLD. It is increasingly recognized that free fatty acids (FFA) and soluble mediators, synthesized from immune cells and adipose tissue, are crucially involved in regulating insulin action and NAFLD occurrence [23,24]. The central role of IR in liver diseases is further suggested by evidence that it is present also in nonobese, nondiabetic subjects with NAFLD [25]. Subjects with NAFLD and IR present an impairment in muscle glucose uptake, an alteration in suppression of hepatic endogenous glucose production induced by insulin [25,26] and a high lipolytic effect in the adipose tissue resulting in an increased FFAs release [27]. The importance of visceral fat in the pathogenesis of hepatic IR and steatosis has been widely demonstrated in preclinical and clinical studies [28]. In particular, in an animal model of inherited leptin resistance, the leptin-receptor-deficient Zucker (*fa/fa*) rat, the surgical resection of intra-abdominal fat depots reverses both hepatic IR and steatosis [29]. In humans, a clear relationship exists between hepatic IR and visceral fat leading to altered adipokine production and increased FFAs [30,31]. The enlargement of the adipose tissue, and in particular visceral fat, has been associated with a tissue inflammation characterized by a decreased release of insulin-sensitizing and anti-inflammatory cytokines, and an increased expression of pro-inflammatory molecules, which modify adipokine secretion [31]. Subjects with NAFLD exhibit decreased adiponectin levels [32], which are correlated negatively with the hepatic triglyceride content. Interestingly, although the three-dimensional structure of adiponectin closely resembles that of TNF-α, these two proteins have completely opposite effects [33]. Both in vivo and in vitro experiments demonstrated that the production and function of adiponectin and TNF-α are inversely correlated in their target tissues [34]. Administration of adiponectin into mice has been shown to produce beneficial effects on lipid metabolism, such as enhancing lipid clearance from plasma and increasing fatty acid β-oxidation in muscle, whereas gluconeogenesis and de novo lipogenesis are decreased in the liver [35].

It has been demonstrated that the insulin-sensitizing effect of adiponectin is mediated by an increase in fatty acid oxidation through sequential activation of AMP kinase, p38 mitogen-activated protein kinase and peroxisome proliferator-activated receptor (PPAR) α [36]. Other adipokines, such as leptin, visfatin and resistin, have also been reported to be involved in hepatic triglyceride accumulation and inflammation. However, the role of these factors and their interplay is still to be elucidated [31].

It is well known that steatosis may interfere with sinusoid microcirculation and hepatocellular clearance of microbial and host-derived danger signals, enhancing responsiveness of Kupffer cells, which critically contribute to progression of NAFLD [37]. Altered lipid

homeostasis in NAFLD negatively affects TLR4 complex assembly and sorting, leading to alternative signaling pathways activation, such as nuclear factor-κB (NF-κB)/AP1 or interferon regulatory factor 3, and promoting differential gene transcription. These differential pathways were found to be similar not only in Kupffer cells and hepatic stellate cells but also in other hepatic nonimmune cell populations, including hepatocytes, biliary epithelial and endothelial cells [18,19].

Additional factors appear to interact with adiponectin to regulate the hepatic triglyceride content. Among these, PPARs that belong to the nuclear receptor superfamily impact on multiple processes involved in lipid trafficking and metabolism, and fuel partitioning [38]. In particular, PPARα regulates mitochondrial and peroxisomal fatty acid β-oxidation pathways by modulating many genes encoding the enzymes involved in these processes (i.e., acyl-CoA synthetase, carnitine palmitoyl transferase I and very-long-chain acyl-CoA dehydrogenase).

Loss or reduction of PPARα expression, in KO mice or in animal fed a methionine–choline-deficient diet or a high-fat diet (HFD), both result in hepatic steatosis [39–41]. In nutritional NAFLD models, the administration of a potent PPARα agonist or probiotics is found to improve the hepatic steatosis. These findings suggest that under conditions of an increased hepatic fatty acid influx, or a decreased hepatic fatty acid efflux, PPARα activation prevents the accumulation of triglycerides by increasing the rate of fatty acid catabolism [41,42].

A growing body of the literature implicates PPARs in the pathogenesis and treatment of NAFLD, linking PPARα and PPARγ to NAFLD/NASH [43]. In fact, PPARγ is expressed at high levels in the adipose tissue and plays a role in increasing insulin sensitivity, as well as in promoting fatty acid uptake into adipocytes [44]. The clear effect of PPARγ activation is the increase in the adipocyte triglyceride storage, thus reducing delivery of fatty acids to the liver. Moreover, PPARγ increases insulin sensitivity by up-regulating glucose transporter 4, an insulin-dependent glucose transporter in the adipose tissue and striated muscle, and by inducing expression of the c-Cbl associated protein, which is involved in insulin signaling [45]. Additionally, in mouse models of IR, PPARγ activation attenuated the induction of suppressor of cytokine signaling 3 (SOCS3), which is involved in the development of IR [46]. PPARγ expression also might reduce the hepatic inflammation by decreasing the expression of proinflammatory cytokines, such as TNF-α [47]. Moreover, adiponectin is up-regulated by PPARγ, thereby providing a connection between the two receptor isotypes.

The complexity and the chronology of pathophysiological events leading to the development of NAFLD/NASH are not fully understood. The increased intrahepatic levels of FFAs provide a source of oxidative stress, which is in part responsible for the progression from steatosis to steatohepatitis and cirrhosis. FFAs may elicit hepatotoxicity by several mechanisms, among others a direct cytotoxic effect [48], an increased lysosomal permeability and TNF-α synthesis by hepatocytes [49]. TNF-α is a pleiotropic cytokine that activates several signaling mechanisms leading to hepatocyte apoptosis, activation of hepatic stellate cells and hepatic inflammatory cell recruitment. TNF-α is also known to inhibit propagation of insulin/insulin receptor-initiated signals by Ser^{307} phosphorylation and Tyr dephosphorylation of the insulin receptor substrate-1 [50]. Therefore, TNF-α represents a crucial protagonist of IR that links the hormonal and metabolic alterations to the inflammatory process. A part TNF-α, IL-6 is another mediator that relates obesity-induced inflammation to IR [51]. High serum IL-6 level is associated with IR and NAFLD [52], and the induction of SOCS3 in the liver may be an important mechanism of IL-6-mediated IR [53]. Finally, the inhibition of TNF-α and IL-6 may limit NASH and/or IR [54].

Recently, we have also demonstrated the involvement of metalloproteinases (MMPs) in the evolution of the liver inflammatory process induced by an HFD [41,55]. These MMPs degrade the basement membrane and extracellular matrix, and facilitate leukocyte migration and the release of TNF-α from its membrane-bound form, thus contributing to steatosis progression.

4. Probiotics, prebiotics and symbiotics

4.1. Probiotics

A probiotic is usually defined as a live commensal microorganism that, when consumed in adequate quantities, confers a health benefit to the host (FAO/WHO 2001). Criteria for designating a commensal strain as a probiotic include a nonpathogenic, human origin; acid and bile resistance; survival of gastrointestinal transit; production of antimicrobial substances; and immune modulator activity [56–59]. The main probiotics on the market are lactobacilli, streptococci and bifidobacteria, which are normal constituents of the human gastrointestinal microflora (Table 1). The first two ones belong to a large group of bacteria designated as LAB [60]. LAB are described as Gram-positive, nonsporing, anaerobic cocci or rods, and traditionally have become associated with the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* [61]. This denomination emphasizes the commercially important aspect of their metabolism, since they produce lactic acid as the major end product during the fermentation of carbohydrates. The genus *Bifidobacterium* is unrelated to LAB phylogenetically, and the *Bifidobacterium* species use a unique metabolic pathway for sugar metabolism. However, they are often considered to be LAB and probiotics because of their documented health-promoting effects [62].

Recent studies have demonstrated that beneficial effects were achieved not only by live bacteria but also by heat-inactivated or gamma-irradiated not viable bacteria, isolated bacterial DNA or even probiotic-cultured media [63], presuming that probiotics can "talk" to immune cells recognizing directly specific receptors or that are otherwise sensitive to probiotic-derived products (e.g., metabolites, cell wall components, DNA). The field instead needs to consider specific immunological applications, whether prophylactic or therapeutic, and then proceed to address mechanisms by which ingested probiotic organisms might be used to prevent or treat several disorders.

4.2. Prebiotics

Prebiotics are indigestible carbohydrates that stimulate the growth and the activity of beneficial bacteria, particularly lactobacilli and bifidobacteria [64]. Many years ago, the prebiotic lactulose has been shown to improve symptoms in liver patients increasing the numbers of bifidobacteria [65], and today it is of common use in these patients [66]. Oligosaccharides that are contained in human milk are considered to be the prototype of prebiotics, since they have been shown to facilitate the growth of bifidobacteria and lactobacilli in the colon of breast-fed neonates [67,68]. Any food that reaches the colon other than nondigestible carbohydrates, such as peptides and proteins, as well as certain lipids, is a potential prebiotic. Fructooligosaccharides (FOS) consist of short- and medium-length chains of β-D-fructans in which fructosyl units are bound by a β 2-1 linkage, with the degree of polymerization varying between 2 and 60 (inulin) or 2 and 20 (oligofructose) [69]. Because of the presence of the β-linkages, FOS are indigestible in the upper gastrointestinal tract. Consequently, they enter the cecum/large bowel as intact, and here they are largely fermented to SCFA (mainly acetate, propionate and butyrrate and other metabolites, e.g., lactate) and cause proliferation of selected anaerobic bacteria, mostly bifidobacteria [69,70]. Thus, FOS including inulin, other oligosaccharides, lactulose, resistant starch and dietary fibers have been shown to promote a probiotic response [64]. Previously, it was also demonstrated that FOS modifying the gene

Table 1
Main probiotics used in commercial preparations

Lactobacilli	*L. acidophilus*, *casei*, *delbrueckii* subsp. *bulgaricus*, *reuteri*, *brevis*, *cellobiosus*, *curvatus*, *fermentum*, *plantarum*, *paracasei*, *rhamnosus* (GG), *salivarius*, *gasseri*, *johnsonii*, *helviticus*, *farciminis*
Bifidobacteria	*B. bifidum*, *infantis*, *longum*, *thermophilum*, *adolescents*, *lactis*, *animalis*, *breve*
Fungi	*Saccharomyces cerevisiae* and *s boulardii*
Others	*Streptococcus thermophilus*, *Enterococcus faecium*, *Lactococcus lactis*, *Propionibacterium freudenreichii*, *Escherichia coli* Nissle 1917, *Bacillus clausii*, *Bacillus oligonitrophilis*

expression of lipogenic enzymes reduced the de novo liver fatty acid synthesis [71], contributing to the decrease in triglyceride accumulation in the liver. A number of studies provide novel insights on the possible link between prebiotics and metabolic diseases, such as obesity and IR [72,73]. Prebiotic supplementation is able to increase plasmatic gut peptide concentrations (glucagon-like peptide 1 and peptide YY), which may contribute in part to the changes in satiety and postprandial glycemic response in healthy subjects [74]. A functional food approach has been utilized to add FOS, primarily inulin, to products (cereals, biscuits, infant foods, yogurts, breads and drinks) or to dietary supplements at concentrations at which a prebiotic effect may occur [75].

Indeed, the modification of intestinal microflora (increase in bifidobacteria and subsequent reduction in Enterobacteriaceae) contributes to a reduction in fecal pH, which results in a minor rate of ammonia absorption and in a lower amount of total ammonia into the bloodstream. Considering all this evidence, it is logical to assume that also the prebiotics would be good candidates to protect the liver in individuals with fatty liver and other liver problems.

4.3. Symbiotics

The term *symbiotic* is used "when a product contains both probiotics and prebiotics" [70]. For example, the symbiotic combination of a specific oligofructose-enriched inulin and *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 for 12 weeks caused a 16% and 18% increase in the numbers of *Lactobacillus* and *Bifidobacterium*, respectively, and a 31% decrease in the numbers of *Clostridium perfringens* [76]. Recent in vitro studies have confirmed that symbiotic was more effective than prebiotics or probiotics in modulating the gut microflora [77].

5. Biological and molecular basis of probiotic action in NAFLD

Clinical and experimental studies suggest that probiotics differ greatly in their effects and mechanisms of action. Significant differences exist, not only among the probiotic species but also within the same strain. The understanding of the various mechanisms of the probiotic action is crucial for the establishment of definitive selection criteria for certain strains or combination of strains in specific clinical conditions. Although the molecular mechanisms of probiotic are not fully elucidated, many effects may result beneficial in NAFLD, including the modulation of the intestinal microbiota, the antibacterial substance production, the epithelial barrier function, intestinal inflammation or the immune system (Fig. 1).

5.1. Modulation of the intestinal microflora composition and antibacterial factor production

Probiotic can limit the role of bacterial pathogens in NAFLD through at least two mechanisms: the exclusion or inhibition of invading bacteria and the production of antimicrobial factors. Nonspecific antimicrobial substances include SCFAs [78], hydrogen peroxide [79], bacteriocins, bacteriocin-like inhibitory substances and bacteriophages [80].

SCFA are produced during the anaerobic metabolism of carbohydrates, especially by strains of lactobacilli, and have an important role in decreasing pH and inhibiting the growth of a wide range of Gram-negative pathogenic bacteria. The inhibition of microbial growth by organics may be due to the ability of these acids to pass across the cell membranes, dissociate in the more alkaline cell environment and acidify the cytoplasm [81]. Alternatively, fermentation acid dissociation in the more alkaline interior causes an accumulation of the anionic species, and this accumulation is dependent on the pH gradient (delta pH) across the membrane and may cause osmotic stress [82]. In microbial fermentor systems, pH modification may lead to a shift in the composition of the microbiota community [83], limiting the populations of certain gut pathogens [84]. Bacteriophages are highly specific and can be active against a single strain of bacteria. The two-component lantibiotics, a class of bacteriocins produced by Gram-positive bacteria, such as *Lactococcus lactis*, are small antimicrobial peptides [85]. These peptides have been found to be active at nanomolar concentrations to inhibit multidrug-resistant pathogens by targeting the lipid II component of the bacterial cell wall [86]. Other non-lanthionine-containing bacteriocins are small antimicrobial peptides produced by lactobacilli. These peptides have a relatively narrow spectrum of activity and are mostly toxic to Gram-positive bacteria, including *Lactococcus*, *Streptococcus*, *Staphylococcus, Listeria* and mycobacteria. The main mechanisms of bacteriocin action are based on forming pores in the cytoplasmic membrane of sensitive bacteria and interfering with essential enzyme activities. In addition, several strains of *Bifidobacteria* have been found to produce bacteriocin-like compounds toxic to both Gram-positive and Gram-negative bacteria [87]. Bifidobacteria and lactobacilli can adhere to intestinal epithelial cells through surface-expressed proteins [88,89]. In particular, *Lactobacillus casei* binds to extracellular matrix components, such as collagen, fibronectin or fibrinogen [90]. Moreover, apart from their antimicrobial effects, some secreted probiotic factors are also able to inhibit the binding of pathogenic bacteria to the specific receptors expressed on the epithelium surface [88]. Several strains of lactobacilli and bifidobacteria are capable to compete with and displace pathogenic bacteria, including *Bacteroides vulgatus, Clostridium histolyticum*, *Clostridium difficile*, *Enterobacter aerogenes*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica*, *Yersinia enterocolitica* [91], enterotoxigenic *Escherichia coli* [92,93] and enteropathogenic *Escherichia coli* [94], even if the pathogens have attached to intestinal epithelial cells prior to probiotic treatment [91]. In this context, recent studies regarding proteinase treatment and carbohydrate competition have confirmed that the probiotic binding to intestinal epithelial cells is mediated by lectin-like adhesion and proteinaceous cell surface components [95,96], which are the same receptors mediating pathogenic bacteria binding to intestinal epithelial cells. For example, lactobacilli and bifidobacteria establish mannose and Galβ1-3GalNAc-specific adhesions to attach to intestinal epithelial cells and mucus [95], competing with pathogens for lectin binding sites of glycoconjugate receptors for intestinal adherence. Therefore, the capability of probiotics to improve gut ecology and microbial composition, in inhibiting pathogenic bacteria growth and/or competing with and displacing pathogenic bacteria, is likely to prevent small intestinal bacteria overgrowth.

5.2. Modification of intestinal epithelial permeability and function

Probiotics are able to improve the nonspecific intestinal barrier defense mechanism, modulating tight junctional protein mucins and stimulating their production. These effects limit small intestinal bacterial overgrowth and bacterial translocation. Both events are

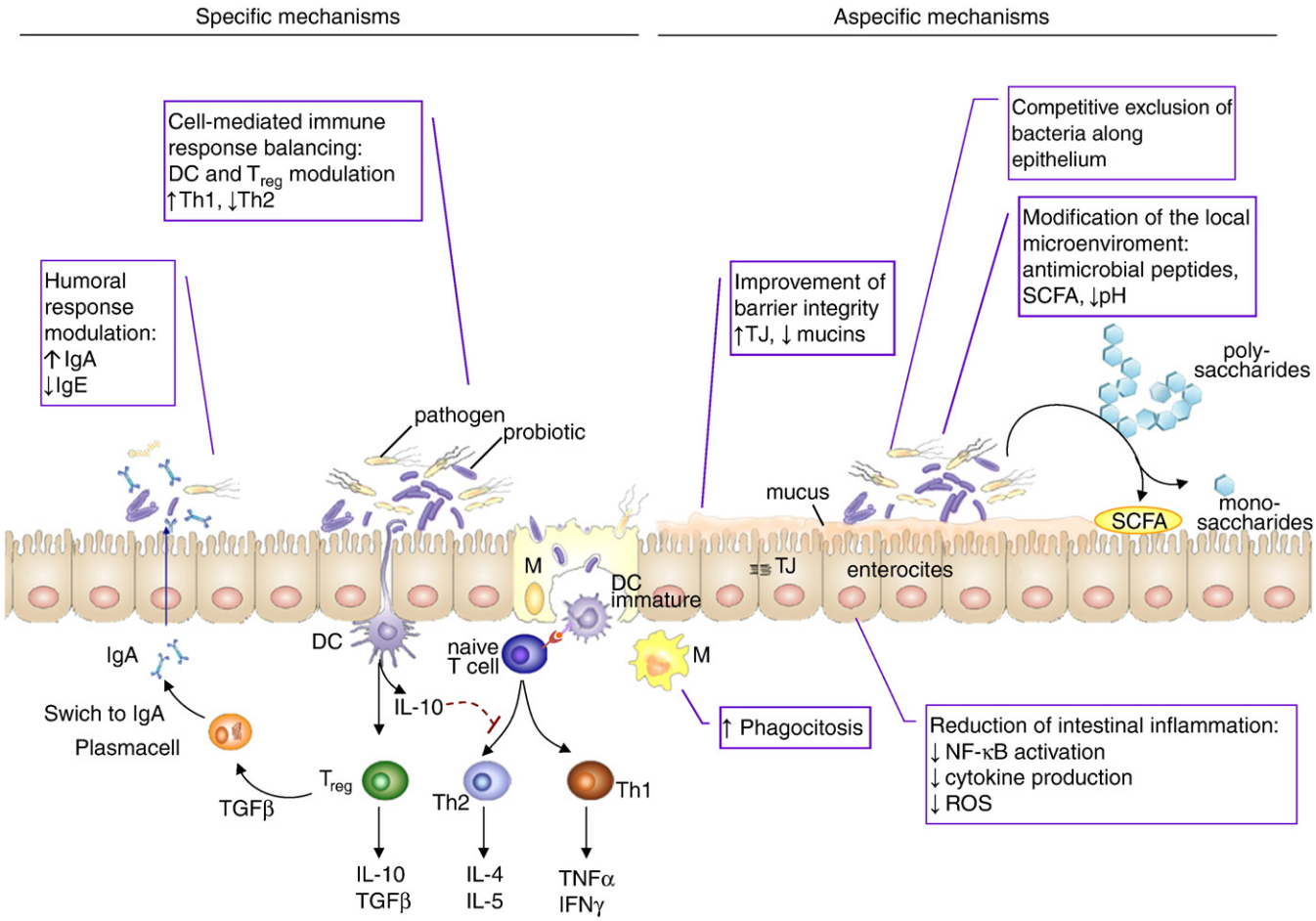

Fig. 1. Mechanisms of action of probiotics. Specific mechanisms: involvement of probiotics in cell-mediated and humoral immune responses. Aspecific mechanisms: enhancement of epithelial barrier function, competitive exclusion of bacteria along epithelium, modification of local microenvironment and reduction of intestinal inflammation. Th, T helper cell; Ig, immunoglobulin; Treg, regulatory T cell; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; IFN, interferon; M, M cell; DC, dendritic cell; TJ, tight junction; MΦ, macrophage; SCFA, short-chain fatty acid; NF-κB, nuclear factor-κB; ROS, reactive oxygen species.

observed in humans and in animal models and are responsible for a reduced endotoxemia [97].

The mucus layer covering the gastrointestinal mucosa is considered the first line of defense against mechanical, chemical or microbiological aggressions arising from the luminal contents. Indeed, the break of the mucus barrier in an inflamed colon has been shown to allow bacterial adherence to the epithelial tissue [98], and the removal of the mucus layer favors the penetration of high-molecular-weight probes in the mucosa [99]. It has been demonstrated that lactobacilli up-regulate the MUC2 and MUC3 mucins and inhibit attachment of enterohemorrhagic *Escherichia coli* in vitro [100], and that a probiotic mixture of lactobacilli and bifidobacteria increase the secretion of mucin, stimulating MUC2 gene expression in the rat colon in vivo [101].

Probiotics stimulate the production of SCFAs [102], which, in turn, are able to modulate intestinal permeability as demonstrated under several conditions, including antibiotic-associated colitis, inflammatory bowel disease, colon cancer and hepatic encephalopathy. Probiotic administration may potentially reduce bacterial metabolites, which may be toxic to the intestinal epithelium, for instance hydrogen sulfide and extracellular superoxide [103].

Lactobacillus GG, *Bifidobacterium infantis*, *Bifidobacterium lactis* and *Escherichia coli* Nissle 1917 increase tight junction integrity, preventing tight junction disruption. The biochemical pathways mediating the probiotic effect on tight junction functions include protein kinase C and mitogen-activated protein kinase pathways, and involve both the redistribution and altered expression of the tight junction proteins occludin, ZO-1 and ZO-2, and claudins 1, 2, 3 and 4 [104,105].

5.3. Modification of endotoxemia

Besides the clear role played by endotoxin levels in alcoholic liver injury, the involvement of endotoxemia in NAFLD has also been addressed. The increase of endotoxemia and the induction of hepatic TLR4 and TLR accessory molecules (MD-2 and CD14) were evidenced in mice fed with a methionine–choline-deficient diet, suggesting that TLR4 signaling is, indeed, important for the pathogenesis of NASH [106]. Moreover, depletion of Kupffer cells lowered diet-induced increases in TLR4 and TNF-α, indicating a crucial role for these cells in mediating TLR4 signaling and transcription of cytokines. Our preliminary data evidenced that rats fed with Surwit diet, a model of IR and NASH, showed an increase in the expression of hepatic TLR4. Indeed, the low physiological levels of these receptors are suggestive of the high tolerance of this organ to intestinal bacteria and bacterial PAMPs recognized as TLR ligands. In this model, a chronic treatment with *Lactobacillus paracasei* (strain B21060) restores the low TLR4 expression in the liver, reducing inflammatory pathways downstream the TLR4 signaling and subsequently delaying NAFLD development (our unpublished data).

5.4. Suppression of inflammation

Intestinal inflammation leads to an increase of mucosal permeability and bacterial translocation. Several cytokines, such as TNF-α, interferon (IFN)-γ, IL-4 and IL-13, have been shown to increase permeability in vitro [107], altering tight junction morphology and distribution [108], thereby creating a self-perpetuating vicious cycle that amplifies bacteria translocation, and possibly, extraintestinal inflammation and damage.

Within intestinal epithelial cells, the transcription factor NF-κB is a master coordinator of immune and inflammatory responses to pathogenic bacteria and other stress signals. However, most commensal bacteria do not activate NF-κB, while some of them are able to antagonize it within enterocytes by several mechanisms. In particular, the nuclear export of the p65 subunit of NF-κB is likely to occur in a PPARγ-dependent manner [109]. Soluble components from a mixture of commercially available probiotics, VSL#3 and *Lactobacillus reuteri* inhibited the epithelial proteasome function, preventing the degradation of IκB [108,110]. This event was accompanied by an increased expression of nerve growth factor, which has anti-inflammatory properties. This finding implicates a role for the enteric nervous system in host microbial interactions.

A few probiotic bacteria, including the mixture VSL#3, *Lactobacillus reuteri*, *Lactobacillus salivarius* UCC118 and *Bifidobacterium infantis* 35624 have been shown to suppress IL-8 secretion from intestinal epithelial cells in response to several pathogenic bacteria [108,111]. This cytokine [112] transcriptionally regulated by NF-κB is a potent neutrophil-recruiting and neutrophil-activating chemokine. The anti-inflammatory effects of a number of probiotic bacteria including *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* UCC118 have been shown also to be mediated, though only in part, via NF-κB [111]. Besides NF-κB pathway, other intracellular signal transduction pathways have also been associated to the protective effects mediated by probiotics. These include mitogen-activated protein kinase, protein kinase B, activator protein-1 and PPAR-γ pathways [113–115].

Apart from intestinal inflammation, small intestinal bacterial overgrowth and translocation result in endotoxemia that directly stimulates hepatic Kupffer cells to produce TNF-α and oxygen free radicals [116,117]. The role of TNF-α in NAFLD has been well documented and was strengthened by the improved liver function with anti-TNF therapy [118,119]. TNF release, in fact, stimulates liver fibrosis and increases lipid peroxidation, contributing to the pathogenesis of fatty liver disease [120,121]. A study performed in *ob*/*ob* mice, as a model of NAFLD, demonstrated an improvement in mice treated with the probiotic mixture VSL#3, also related to a reduction of TNF-α activity [119]. Similar data were obtained by our group in a model of NAFLD induced by an HFD: we demonstrated the antioxidative and anti-inflammatory effect elicited by VSL#3 in an experimental model of NASH induced in young rats. This probiotic mixture induced a decrease in the oxidative stress, evidenced through the reduction of malondialdehyde, and protein nitrotyrosilated levels in the liver. Moreover, VSL#3 exhibited an anti-inflammatory activity by a reduction of NF-kB activation in the liver and, hence, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) expression. This effect was also evidenced by VSL#3 capability to reduce hepatic TNF-α level, the key pathogenetic factor responsible for the onset of NASH, and for restoring PPARα expression [41]. Another study measured the hepatic natural killer T (NKT)-cell depletion in high-fat-fed animals. This diet induced the depletion of NKT from the liver, leading to overproduction of TNF-α and causing inflammation, IR and steatosis. VSL#3 significantly improved all these parameters restoring insulin signaling [122]. Considering the anti-inflammatory properties of more than 550 different LAB strains, a new symbiotic composition was obtained, consisting of *Lactobacillus plantarum*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactococcus raffinolactis* and *Pediococcus pentosaceus*, plus four different fibers known for their strong bioactivity: betaglucan, inulin, pectin and resistant starch. This composition, Symbiotic 2000, was successfully investigated in surgical operations such as liver transplantation, reducing the problem of postoperative infections [123].

5.5. Immune system modulation by probiotics

Commensal bacteria can modulate the immune system at both local and systemic levels. Signals mediated by these bacteria are essential for optimal mucosal and immune development, and to maintain or restore gut integrity [124,125]. In the intestinal tract, immunocytes, such as enterocytes, M cells and dendritic cells (DCs), are constantly responding to intestinal bacteria. These cells express pattern recognition receptors, such as TLRs, that engage bacterial signals (LPS, lipotechoic acid, bacterial DNA and flagellin) and contribute to the activation of transcription factors and proinflammatory cascade. Immune engagement and systemic immunologic changes are associated with oral consumption of probiotics [126], which share the same host–microbial signaling pathways of commensal microbiota. In the intestine, probiotic bacteria are internalized by M cells to interact with DCs and follicle-associated epithelial cells, initiating responses mediated by macrophages and T and B lymphocytes [127].

DCs initiate immune responses in vivo by presenting antigens to T cells and influence polarization of T-cell responses (Th1, Th2, Th3 or regulatory T cells) through secretion of immunoregulatory cytokines. Moreover, DCs contribute to oral tolerance induction by generating regulatory T cells and IgA-producing B cells through production of cytokines, such as IL-10 and transforming growth factor (TGF)-β [128].

Regulatory T cells produce high levels of IL-10 and suppress the proliferation of effector T cells in an IL-10-dependent manner. Different strains of lactobacilli and other probiotic bacteria can modulate DCs function modulating cell maturation and the expression of regulatory cytokines, such as IL-10 [129,130].

DCs from different lymphoid compartments exhibit divergent cytokine responses to probiotic and pathogenic bacteria [131]. Some strains of probiotic bacteria, such as *Lactobacillus casei* or *Lactobacillus reuteri*, but not *Lactobacillus plantarum*, can promote DCs to induce tolerance driving the development of regulatory T cells [132]. Similarly, VSL#3 can ameliorate Th1 cell-mediated murine colitis, by restoring cytokine balance through the induction of IL-10- and TGF-β-bearing regulatory T cells [133].

Probiotics can interact either directly with DCs or indirectly, via the action of M cells. Very recently, the ability of three lactobacilli strains (*plantarum*, LGG and *paracasei* B21060) to activate DCs has been evaluated. *Lactobacillus paracasei* B21060 has been identified as the more immunomodulatory among the three strains, being able to inhibit the inflammatory potential of pathogenic *Salmonella* and to protect against experimental colitis [134].

Probiotics, in addition to facilitating cell-mediated immunity, are able to promote humoral response. The administration of probiotic bacteria leads to an increase in the levels of pathogen-specific IgA [135], and IgA responses are enhanced in formula-fed infants supplemented with probiotics as compared with infants receiving placebo [136]. Noteworthy is the induction of IgA in the gut being heavily dependent on TGF-β, and also closely involved in the maturation of regulatory T cells [137]. In agreement with these studies, a recent randomized, double-blind, placebo-controlled trial has demonstrated that the administration of two probiotic bacteria, *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis*, increased the proportion and activity of phagocytic and NKT cells, as well as the levels of IgA in healthy adults [138,139].

Particularly desirable strains are those that improve the immune function by increasing the number of IgA-producing plasma cells, as well as improving phagocytosis, and the proportion of Th1 cells and NKT cells [140]. Some strains are more likely to have strong clinical effects; among them are strains like *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*. In particular, *Lactobacillus paracasei* has been shown to induce cellular immunity and stimulate production of suppressive cytokines such as TGF-β and IL-10, to suppress Th2 activity and CD4 T cells [141], as well as splenocyte proliferation [142], and to decrease antigen-specific IgE and IgG1 [143]. *Lactobacillus paracasei* was also shown to be the strongest inducer of Th1 and repressor of Th2 cytokines [144]. Moreover, co-culturing LAB with human or rodent leukocytes has been shown to augment the production of type II IFN-γ by mitogen-stimulated mononuclear cells, or to induce type I IFN-α production by isolated macrophages [145,146]. Both interferons promote Th1-type immune responses and reduce IgE production [147].

IL-12 has been shown to be an important pro-interferon cytokine involved in the production of LAB-stimulated IFNγ [146]. IL-12 is known to be an effective cytokine during the early differentiation of Th0 cells, promoting development of Th1 lymphocytes and augmenting NKT cell function; both of these actions increase IFNγ-producing capacity, limiting the overexpression of a Th2 phenotype. Moreover, IL-12 has also been demonstrated to regulate IL-4 production, limiting both the establishment and maintenance of Th2-type responses [148].

In vitro studies have indicated that LAB are potent stimulants for IL-12 production by intestinal mucosa or peripheral blood leukocytes [149,150]. In addition, some lactobacillus strains stimulate the production of IL-18 by human leukocytes [149]. In its turn, IL-18 acts synergistically with IL-12 to enhance IFNγ production and to promote a Th1 phenotype [151]. Thus, the presumed scenario is that immunoregulatory LAB stimulate the production of pro-interferon monokines (IL-12 and IL-18) which, in conjunction with IFN-α, induce production of IFNγ; this biases a developing T lymphocyte-mediated immune response toward a Th1 phenotype and, more interestingly, away from a pro-allergy Th2 phenotype [135].

The varying immunological effects of bacteria highlight the differences arising when different cellular, fluid or tissue systems are used. However, there appear to be different responses of different bacterial strains even within one genus. All these observations need to be considered to properly address the immunomodulation capacity of probiotics.

6. Probiotic efficacy in NAFLD: from animal models to clinical evidences

The major difficulties in our knowledge about probiotics efficacy in NAFLD derived from the different experimental models used and bacterial strains tested (Table 2). Clinical research into mechanisms of NAFLD development and progression is restrained by ethical considerations, particularly with respect to obtaining liver and other tissues, and by inadequate ability to delineate cause and effect from complex pathology because of the many mechanisms involved. From an experimental viewpoint, it is, therefore, attractive to use animal models. Research models of NAFLD may be divided into two main typologies, those caused by genetic mutation and those with an acquired NAFLD phenotype [152–154].

The central feature of the "modern lifestyle" that predisposes to overweight, obesity, IR and fatty liver disease is the constant caloric overconsumption, also known as "overnutrition." The latter has been achieved in animal models in a number of different ways, including forced feeding, administration of HFDs, the use of genetically hyperphagic animals or a combination of these approaches. The effects of administering an HFD to rodents can be highly variable based on treatment duration, animal strain, percentage and nature of fat added to diet.

The high percentage of fat contained in the diets may range between 40% and 70%. The well- known study by Lieber and colleagues [155] described the effects of feeding a liquid HFD to Sprague–Dawley rats. High-fat-fed rats showed quickly extensive mitochondrial abnormalities and dysfunction producing reactive oxygen species with an array of responses that resulted in hepatocyte injury and cell death, inflammation and fibrosis. Conversely, to better study the relationship between the visceral adipose tissue and the liver, it is possible to use a high-fat and calorie-solid diet [156], by creating in several weeks a model of IR and NAFLD/NASH in nongenetically modified animals [157]. This model is characterized by visceral obesity, increased glucose and insulin levels, decreased PPARα expression, and alterations in insulin signaling and hepatic steatosis, leading to oxidative stress, necroinflammatory liver injury, cell apoptosis and collagen deposition. On the other hand, different diet manipulations have been shown to induce obesity and fatty liver in a number of different strains and species of rodents, suggesting that "overnutrition" with either carbohydrates (fructose and sucrose) or fats (fatty acid and cholesterol) or both might play a role in the genesis of obesity-related NAFLD.

The efficacy of probiotics in several experimental models of NAFLD/NASH is reported in Table 2. As depicted, the most characterized probiotic is VSL#3 mixture, active in several murine models of HFD-induced NAFLD/NASH [41,119,122].

Li et al. [119] using *ob*/*ob* mice fed with an HFD provided first evidence that manipulation of the intestinal flora in this experimental model influences obesity-related fatty liver disease. In fact, VSL#3 similarly to anti-TNF-α antibodies improved liver histology, reduced hepatic total fatty acid content and decreased serum alanine aminotransferase (ALT) levels. These effects were associated with a reduction in Jun N-terminal kinase and NF-κB activity, fatty acid β-oxidation, and mitochondrial uncoupling protein-2 expression, all being markers and factors characterizing IR. Subsequently, Ma et al. [122] showed that oral VSL#3 treatment significantly improved the HFD-induced IR and steatosis recovering hepatic NKT cell depletion. Our research group also showed the efficacy of VSL#3 in NAFLD [41]. In our study, the VSL#3 was able to ameliorate lipid profile and reduce inflammation and oxidative damage, protein nitrotyrosilation, and tissue TNF-α level, interfering with the key pathogenetic mechanisms responsible for the onset of liver damage. We also demonstrated a direct effect of VSL#3 in reducing inflammatory enzymes, such as iNOS and COX-2, and restoring PPAR-α. The VSL#3 treatment also reduced hepatic gelatinase activity of proMMP-2 and proMMP-9 in HFD-fed rats [158]. Conversely, recent data have demonstrated that in another model of NAFLD/NASH, VSL#3 attenuated fibrosis, reducing TGF-β and collagen, α-SMA, MMPs expression but had no effect on liver steatosis parameters and inflammation in methionine–choline-deficient diet-fed mice [159]. These data are limited depending on the type of diet used in these animal models. The major drawback of the methionine–choline-deficient diet model is that of being associated with significant weight loss, low serum leptin level and peripheral insulin sensitivity. The severe atrophy of adipose tissue in methionine–choline-deficient diet-fed mice suggests that in this model NASH reflects the associated lipodystrophy rather than the metabolic syndrome [160].

Among probiotics, several strains of lactobacillus have shown to have a protective effect on NAFLD [161–163]. In particular, an 8-week oral treatment with *Lactobacillus rhamnosus* PL60 showed an antiobesity effect and liver steatosis in diet-induced obesity mice. Histopathological analysis of liver steatosis evidenced a lowered grading score in diet-induced obesity mice receiving *Lactobacillus rhamnosus* [162].

Moreover, a beneficial effect on liver alteration has been shown in *Lactobacillus acidophilus* and *Lactobacillus casei*-treated mice fed with

Table 2
Effect of several probiotics in experimental models of NAFLD

Probiotic	Experimental model	Duration of therapy	Results	Reference
VSL#3 1.5×10^9 CFU/mouse/day	Mice: *ob*/*ob* mice fed HFD	4 weeks	Improved NAFLD histology and reduction in hepatic total fatty acid content, and serum ALT levels; amelioration of hepatic IR	[Li et al., 2003 [119]
Bacillus polyfermenticus SCD 3.1×10^6 CFU/day	Rats: high-fat and high-cholesterol diet	6 weeks	Reduction in plasma LDL, cholesterol, and hepatic total cholesterol, and triglycerides	[Paik et al., 2005 [164]
Lactobacillus rhamnosus PL60 1.0×10^7–1.0×10^9 CFU/mouse/day	Mice: HFD	8 weeks	Resolution of hepatic steatosis (at higher dose)	[Lee et al., 2006 [162]
Lactobacillus acidophilus and *Lactobacillus casei*	Rats: high-fructose diet	8 weeks	Reduced liver oxidative stress, improved IR	[Yadav et al., 2007 [163]
VSL#3 1.5×10^9 CFU/mouse/day	Mice: HFD	4 weeks	Improved HFD-induced hepatic NKT cell depletion, IR, hepatic steatosis and inflammation	[Ma et al., 2008 [122]
Lactobacillus plantarum MA2 1×10^{11} CFU/rat/day	Rats: cholesterol-enriched diet	5 weeks	Reduction in liver and serum cholesterol and triglycerides	[Wang et al.,2009 [161]
VSL#3 1.3×10^{10} CFU/kg	Rats: HFD	4 weeks	Amelioration of the hepatic inflammatory, steatotic and peroxidative factors and reduction in serum aminotransferase levels	[Esposito et al., 2009 [41]
VSL#3 in drinking water	Mice: MCD	9 weeks	No effect on MCD-induced liver steatosis and inflammation, but amelioration of liver fibrosis	[Velayudham et al., 2009 [159]
Lactobacillus paracasei B21060 2.5×10^8 bacteria/kg/diet	Rats:	5 weeks	Ameliorated steatosis, IR and decreased hepatic inflammatory cytokines	Our unpublished data

MCD, methionine–choline-deficient.

a high-fructose diet. This diet does indeed provide a dietary model of type 2 diabetes associated with IR, hyperinsulinemia and hypertriglyceridemia. Concomitantly, this overload of fructose to the liver impairs the glucose metabolism and uptake pathways, leading to an enhanced rate of de novo lipogenesis and inducing steatosis. In this study, the two probiotics reported above delayed the onset of glucose intolerance, reduced insulinemia and liver glycogen, and ameliorated steatosis, reducing malonyldialdehyde and increasing gluthathione content [163]. Using a cholesterol-enriched diet, Wang et al. [161] demonstrated that the administration of *Lactobacillus plantarum* MA2 in rats, beyond the hypolipidemic effect, reduced both liver cholesterol and triglycerides, and increased the number of fecal lactobacilli and bifidobacteria. Similar data had been previously observed when *Bacillus polyfermenticus* was administered in rat fed with high-fat and high-cholesterol diet [164]. Recent unpublished data by our laboratory support the beneficial effect of the symbiotic formulation, named FLORTEC, containing viable lyophilized *Lactobacillus paracasei* B21060 mixed with prebiotics (fructo-oligosaccharides and arabinogalactane) on HFD-induced steatosis in young rats, improving metabolic and inflammatory alterations.

Findings obtained so far suggest that probiotics may interfere with the development of NAFLD/NASH at various levels (Fig. 2).

Despite the large number of preclinical studies about the use of probiotics in the treatment of fatty liver disease, there are only two pilot studies concerning their efficacy in NAFLD in humans (Table 3). The first study [121] tested a mixture of probiotics (*Lactobacillus acidophilus*, *bifidus*, *rhamnosus*, *plantarum*, *salivarius*, *bulgaricus*, *lactis*, *casei*, *breve*) associated with prebiotics (FOS) and vitamins (B_6, B_2, B_{12}, D_3, C and folic acid) in 10 patients with biopsy-proven NASH. After 2 months of treatment, the treated patients showed a significant improvement of liver damage and function tests, as well as a partial persistence of the effect also after the end of treatment. Another pilot study was carried out to evaluate the effects of probiotic therapy in patients with chronic liver diseases [165]. Four groups of patients were enrolled in the study: 22 NAFLD and 20 alcoholic liver cirrhosis (AC) patients were compared to hepatitis C virus-positive patients with chronic hepatitis, with and without liver cirrhosis. All patients were treated for 3 months with VSL#3. In NAFLD and AC groups, VSL#3 significantly improved plasma levels of malonyldialdehyde and 4-hydroxynonenal, both markers of lipid peroxidation, whereas cytokines (TNF-α, IL-6 and IL-10) were reduced only in AC patients. *S*-Nitrosothiols plasma levels were improved at the end of treatment in all groups. These promising preliminary results are strongly indicative of a great potential for the use of probiotics in the prevention and treatment of NAFLD. However, as recently stated in a Cochrane meta-analysis, further clinical studies are necessary to better define this innovative strategy [166]. The large amount of experimental data on probiotics effects that are nowadays available will very likely drive the design of clinical trials in the next.

7. Adverse effects of probiotics

Probiotics are generally regarded as safe. Side effects are rarely reported and generally amount to little more than flatulence or changes in bowel habits. A review outlining the safety of current probiotic compounds has been published recently [167]. The use of probiotics in immunocompromised or in critical ill patients should be carefully evaluated to limit the risk of endocarditis or sepsis.

However, cases of infection caused by lactobacilli and bifidobacteria are extremely rare and are estimated to occur in approximately 0.05–0.4% of all cases of infective endocarditis and bacteremia [167].

One important clinical characteristic of lactobacilli is their resistance to antibiotic vancomycin, empirically used against Gram-negative bacteremia. Lactobacilli are considered as emerging pathogens in high-risk patients with neutropenia induced by chemotherapy [168], in neonates submitted to surgery on a count of cardiovascular disorders in pediatric patients submitted to gastrojejunostomy [169].

No increase in bacteremia caused by *Lactobacillus* species was seen in Finland over the period of 1990–2000 despite an increased consumption of *Lactobacillus rhamnosus* GG. A study on a long-term consumption of *Bifidobacterium lactis* and *Streptococcus thermophilus*-supplemented formula in children aged less than 2 years showed that the product was well tolerated [170]. Complications of treatment with probiotics have been observed in patients who are immunocompromised or in the intensive care setting. *Saccharomyces cerevisiae* fungemia [171] and *Lactobacillus* bacteremia [169,172] have been reported in patients with severe underlying illnesses. Nevertheless, case reports have identified fungemia in two immunosuppressed patients [171] and exacerbation of diarrhea in two patients with ulcerative colitis who consumed *S. boulardii* [173].

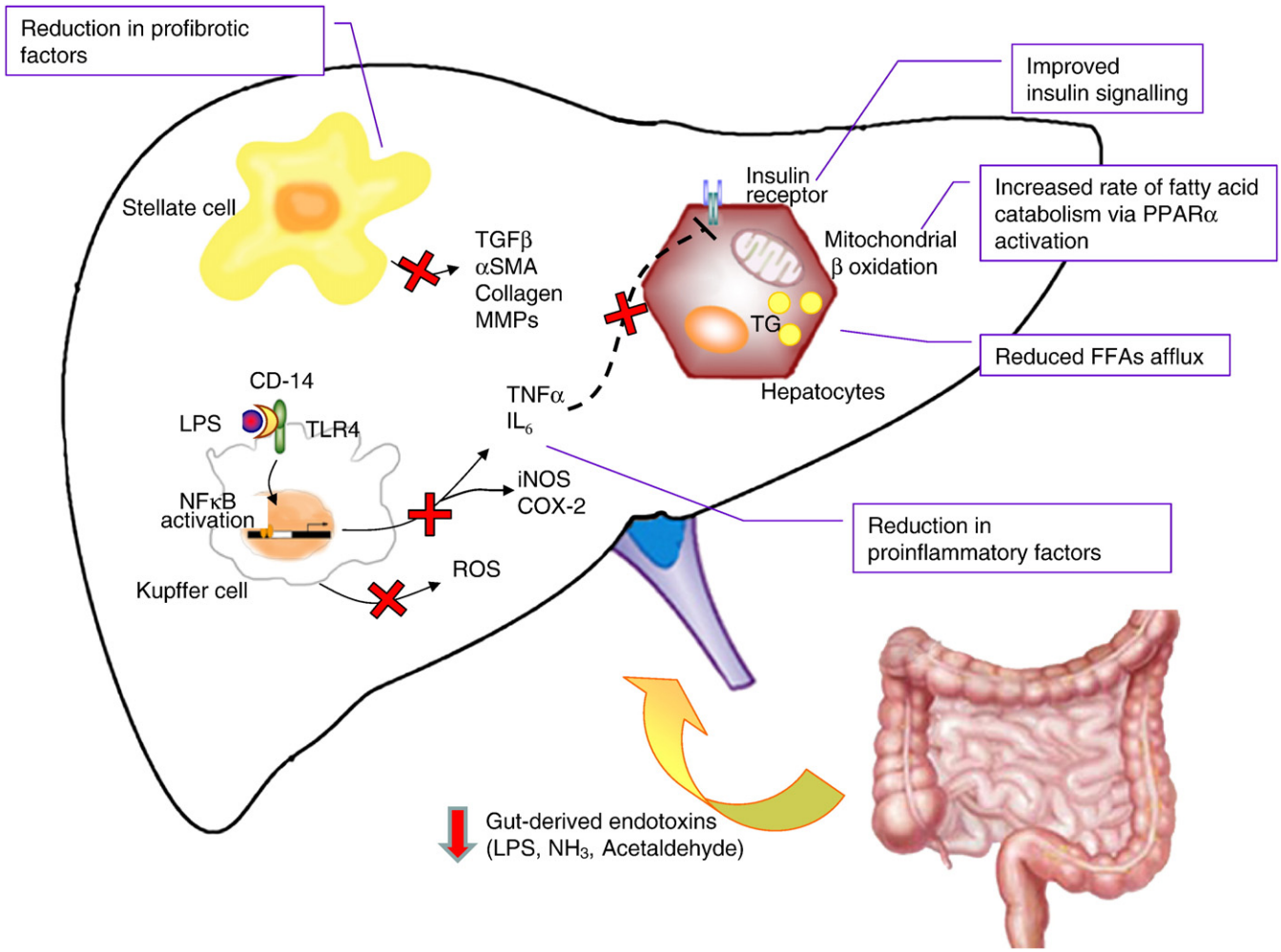

Fig. 2. Cellular mechanisms of probiotics in the liver. The reduction of gut-derived endotoxins leads to a decrease of Kupffer cell stimulation of TLR4 receptor and NF-κB-related gene transcription, with a reduction of inflammation. Probiotics induce a reduction in profibrotic factors by stellate cells, improve insulin signaling, increase the rate of fatty acid catabolism following PPARα activation and reduce FFAs afflux (see red cross). LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; TLR, toll-like receptor; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; SMA, smooth muscle actin; MMPs, metalloproteinases; TG, triglycerides; PPAR, peroxisome proliferator-activated receptor; FFAs, free fatty acids.

8. Conclusions

The study of intestinal microbiota composition and role in different pathological conditions has greatly helped our understanding on the potential use of probiotics in liver diseases, from simple steatosis to cirrhosis. What is now clear is that not all probiotics may have the same effect. High-quality preclinical studies and few randomized controlled trials support the therapeutic use of probiotics in liver diseases. Unfortunately, these data could not be extrapolated for all probiotic compounds now available on the market. The

Table 3
Clinical studies of probiotics on NAFLD

Probiotic	Design	Duration of therapy	Results	Reference
LAB associated to prebiotics (FOS) and vitamins (B_6, B_2, B_{12}, D_3, C and folic acid)	Prospective, single-center, nonrandomized, noncontrolled study pilot study. Three groups of patients: (1) n=12 patients with CHC (2) n=10 patients with AC (3) n=10 patients with NASH	2 months	Decreased serum ALT, γ-GT, MDA, 4-HNE and TNF-α in NASH patients	[Loguercio et al., 2002 [165]
VSL#3	Four groups of patients: (1) n=22 NAFLD (2) n=20 AC (3) n=36 HCV-+ patients (in which n=20 CHC and n=16 CC) liver cirrhosis	3 months	In NAFLD and AC groups, VSL#3 improved plasma levels of lipid peroxidation markers: MDA, 4-HNE. In AC patients, cytokines (TNF-α, IL-6 and IL-10) improved. *S*-NO plasma levels improved in all groups.	[Loguercio et al., 2005 [121]

CHC, chronic hepatitis C; CC, liver cirrhosis; NASH, patients with biopsy-proven nonalcoholic steatohepatitis; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; *S*-NO, *S*-nitrosothiols. LAB mixture contains *Lactobacillus acidophilus*, *bifidus*, *rhamnosus*, *plantarum*, *salivarius*, *bulgaricus*, *lactis*, *casei*, *breve*. VSL#3 mixture contains *Streptococcus thermophilus*; *Bifidobacterium breve*, *longum*, *infantis*; *Lactobacillus acidophilus*, *plantarum*, *casei*, *bulgaricus*.

rationale of the use of mixtures of bacteria is based on the possible combination of different mechanisms of action of individual strains. Additional carefully designed, mechanistic-based laboratory and clinical studies need to be undertaken to provide scientific evidence for the efficacy in NAFLD therapy of probiotics alone or in appropriate synergistic combination between strains or with some prebiotics, that is, lactulose. Keeping in mind "primum non nocere," in the future, nutrients containing pre-probiotics will very likely be considered a new nutritional approach in NAFLD patients.

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