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THERAPEUTIC EFFICACY OF PROBIOTICS AND SHORT CHAIN FATTY ACIDS IN GASTROINTESTINAL DISEASE: EVALUATION OF METABOLIC, HORMONAL AND INFLAMMATORY PARAMETERS

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SUMMARY

INTRODUCTION

In recent years, colonic health has been increasingly linked to maintain the well being and to reduce the risk of various diseases due to changes in diet and lifestyle. At the forefront it has been considered the functional foods, such as ‘‘prebiotics’’ and ‘‘probiotics,’’ dietary fibers, and other dietary components that target the colon and affect its environment, enhancing short chain fatty acids (SCFAs) production, and the conversion of bioactive substances such as phytoestrogens to promote or retard their absorption from the colon. This is far from the classic view that the role of the human colon is to absorb salt and water and to provide a mechanism of the disposal of waste products. The development of the intestinal ecosystem is crucial for gastrointestinal functions and body health. The intestinal ecosystem essentially comprises the epithelium, immune cells, enteric neurons, intestinal microflora, and nutrients. The coordinate interplay between all these components has been the object of intensive research efforts to design new strategies for many intestinal and extraintestinal diseases. In the gut live about 10^{14} bacterial cells, including up 2000 species dominated by anaerobic bacteria .

Intestinal microflora benefit from constant nutrient flow, stable temperature and 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, saving energy from unabsorbed food, producing SCFAs, inhibiting the growth of pathogens, sustaining intestinal barrier integrity, maintaining mucosal immune homeostasis, and participating to the xenobiotic metabolism system [1,2].

Probiotics are live microbes able to modulate the intestinal microflora and enhance body heath. At birth, the gastrointestinal tract is a sterile environment. Within a few months after birth, a relatively stable microbial population is established [2,3]. This abundant, diverse and dynamic microflora normally lives in a complex, synbiotic relationship with the eukaryotic cells of the intestinal mucosa. Firmicutes are the most representative bacteria among phyla found in the human colon, and include Clostridiales and lactic bacteria (LAB), and Bacteroidetes [4]. However, several factors, such as age, diet, hygienic habit, infection and antibiotic therapy can modify microbiota composition. Recently, gut microbiota has been considered as a regulator of energy homeostasis and ectopic fat deposition, evidencing its implications for metabolic diseases [5,6]. 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 [7]. Moreover, high–fat fed animals present gut microbiome with increased transport proteins and

enzymes involved in import and fermentation of simple sugars and host glycans. In return, these substances can be more utilized for hepatic lipogenesis increasing the capability of hosts to harvest energy from diet [8]. Moreover, in healthy subject the microbiota suppresses expression of fasting-induced adipocyte factor (*Fiaf*, also known as angiopoietin-like protein 4), a lipoprotein lipase inhibitor (LPL), produced not only by the intestine but also by liver and adipose tissue, and therefore is an important regulator of peripheral fat storage [9]. So it has become clear that overnutrition alters the immune system and functions. Chronic overnutrition leads to accumulation of fat in adipose tissue, in which subsequently immune cells infiltrate. This gives rise to a mild and sustained increase in inflammatory mediators in the systemic circulation, for example, the acute-phase response marker C-reactive protein (CRP) and its major regulators interleukin IL-6 and tumor necrosis factor TNF-α [10]. In addition, repeated exposure to increased concentrations of proinflammatory cytokines in the postprandial period after high-fat meals or rapidly digestible carbohydrates has recently postulated to be involved in the development of low-grade inflammation in susceptible persons [11,12]. Chronic low-grade inflammation is associated with an increased risk of, among others, insulin resistance (IR), diabetes type 2, and atherosclerosis [13]. For this reason, strategies to suppress low-grade inflammation as a preventive measure for these chronic diseases are intensively investigate.

Food and food-derived substances receive increasing attention as potential factors that can modulate cells or cell functions that play a role in immunological processes. Dietary fiber intake has been shown in prospective studies to be inversely related to plasma concentrations of CRP [14,15] and the proinflammatory cytokine IL-6 [16]. In a human study on healthy elderly, immunomodulatory effects of the prebiotic B-galactooligosaccharides (B-GOSs) were demonstrated [17]. B-GOS significantly increased the production of the anti-inflammatory cytokine IL-10 and significantly reduced production of IL-6, IL-1β, and TNF-α [17]. Another intervention study on fructooligosaccharides in older persons resulted in decreased IL-6 mRNA expression in peripheral blood monocytes [18]. In healthy young individuals, an evening meal rich in nondigestible carbohydrate prevented the glucose-induced postprandial rise in plasma IL-6 and TNF-α concentrations [19]. One of the proposed factors that could explain these effects is the increase in short-chain fatty acids acetate, propionate, and butyrate, which are produced by the colonic microbiota when dietary fiber are fermented [20,21]. SCFAs are rapidly absorbed from the colonic lumen and partly metabolized by colonic epithelial cells. A fraction also enters the portal and peripheral circulation. In six nonfasted sudden death victims, portal concentrations of acetate, propionate, and butyrate lay between 108 and 404 mmol/l, 17 and 194 mmol/l, and 14 and 64

mmol/l, respectively and peripheral concentrations between 19 and 146 mmol/l, 1 and 13 mmol/l, and 1–12 mmol/l, respectively [22]. SCFAs, and especially butyrate, have been for long time, at the center of interest for modulating inflammatory response in the colonic epithelial cells and results of these studies indicate beneficial effects. Therefore, it seems worthwhile to explore whether SCFAs could also affect systemic inflammation. This is especially interesting because, as discussed below, recent studies suggest that obesity induced inflammation is partly antigen-dependent. Information about, for instance, the capacity of SCFAs to reduce activation of T cells by monocyte-presented antigens is, thus, highly relevant in this context. Butyrate irrigation (enema) has also been suggested in the treatment of colitis. More human studies are now needed, especially, given the diverse nature of carbohydrate substrates and the SCFA patterns resulting from their fermentation. Short-term and long-term human studies are particularly required on SCFAs in relation to markers of cancer risk. Taken togrther these considerations, probiotics and SCFAs have been proposed in the treatment and prevention of many conditions. The mechanisms of these effects are multiple, the vast majority are related to the regulation of immune system. Given the close anatomical and functional correlation between the bowel and the liver, and the immunoregulatory effects elicited by these compounds, the aim of this study is evaluate the effects of probiotics and short chain fatty acids in some gastrointestinal disease like non-alcoholic fatty liver disease (NAFLD) and ulcerative colitis (UC), focusing their molecular and biochemical mechanisms and highlighting their efficacy as emerging therapeutic strategy to treat or prevent these conditions.

CHAPTER 1: NALFD

Nonalcoholic fatty liver disease (NAFLD) includes a wide spectrum of diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma [23]. The majority of patients with NAFLD are overweight or even morbidly obese and often shows insulin resistance [24,25]. The proportion of patients with NAFLD who develops NASH is still not entirely clear but might range from 10%-20%. This is relevant because inflammation and/or fibrosis determine the long-term prognosis of this disease, whereas steatosis per se might not adversely affect outcome [26,27]. Most studies indicate that 1%-3% of the Western population might have NASH.

The natural history of NAFLD is still poorly understood, and in particular, it is not known why any patients progress toward inflammation, fibrosis, and cirrhosis and why others do not. One of the burning questions in NAFLD remains the identification of those factors that drive forces toward a more progressive, inflammatory disease phenotype. Day and colleagues presented more than a decade ago the so-called ‘‘two hit’’ model, suggesting that after a first hit (i.e., hepatic steatosis) another hit (e.g., gut-derived endotoxin) is needed to develop NASH [28]. Because simple hepatic steatosis is a benign process in the majority of patients, NASH might be a separate disease with a different pathogenesis. Later, Tilg and Moschen propose a new model suggesting that many hits may act in parallel, finally resulting in liver inflammation and that especially gut-derived and adipose tissue–derived factors may play a central role in steatosis progression. Inflammation may also precede steatosis in NASH, as inflammatory events may lead to subsequent steatosis. Furthermore, the authors highlight the potential importance of endoplasmic reticulum (ER) stress in various aspects of this disease [29].

The majority of patients with NAFLD is overweight or obese, and there is convincing evidence that NAFLD is a component of the metabolic syndrome [30]. NAFLD is currently the most common liver disease worldwide, both in adults and children. NAFLD is characterized by aberrant lipid storage in hepatocytes (hepatic steatosis), and inflammatory progression to non alcoholic steatohepatitis (NASH). Even if pathologically, several patterns of disease exist which resemble alcoholic liver disease, the *sine qua non* condition for NAFLD recognition is hepatic macrovesicular steatosis or fatty liver. Simple steatosis remains a benign process in most affected persons and seem to be well tolerated [26,31]. However, some patients develop superimposed necroinflammatory activity with a nonspecific inflammatory infiltrate, hepatocyte ballooning with

Mallory's hyaline which are the driving force for the development of fibrosis, observed in nonalcoholic steatohepatitis (NASH) [32]. Likely a minority of these patients develops cirrhosis, which may become complicated by hepatocellular carcinoma.

Beginning of hepatic steatosis

A fatty liver is the result of the accumulation of various lipids [33]. Several mechanisms may lead to a fatty liver: (1) increased free fatty acids (FFAs) supply due to increased lipolysis from both visceral/subcutaneous adipose tissue and/or increased intake of dietary fat; (2) decreased free fatty oxidation; (3) increased *de novo* hepatic lipogenesis (DNL) and (4) decreased hepatic very low density lipoprotein–triglyceride secretion [34]. Free fatty acid delivery to the liver accounts for almost two-thirds of its lipid accumulation [35]. Elevated peripheral fatty acids and DNL therefore predominantly contribute to the accumulation of hepatic fat in NAFLD. Besides the well-established lipogenesis-controlling factors such as sterol regulatory element- binding protein (SREBP) or carbohydrate response element-binding protein (ChREBP), X-box binding protein 1 (XBP1), known as a key regulator of the unfolded protein response (UPR) secondary to ER stress, is a only recently characterized regulator of hepatic lipogenesis [36]. Triglycerides are the main lipids stored in the liver of patients with NAFLD. Although large epidemiological studies suggest triglyceride-mediated pathways might negatively affect disease [37], recent evidence indicates that trigylcerides might exert protective functions. Diacylglycerol acyltransferase 1 and 2 (DGAT1/2) catalyze the final step in triglyceride synthesis. In a model of diet induced obesity, mice with overexpression of adipocyte and macrophage DGAT1 are protected from systemic inflammation and insulin resistance by the prevention of macrophage activation and their accumulation in white adipose tissue [38]. Inhibition of triglyceride synthesis via DGAT2 antisense oligonucleotides improves liver steatosis but worsens liver damage, also suggesting that accumulation of liver triglycerides could be a protective mechanism [39].

Hepatic steatosis (i.e., triglyceride accumulation) is dissociated from insulin resistance in patients with familial hypobetalipoproteinemia, providing further evidence that increased intrahepatic triglyceride content might be more a marker rather than a cause of insulin resistance. In summary, triglyceride synthesis seems to be an adaptive, beneficial response in situations where hepatocytes are exposed to potentially toxic triglyceride metabolites. Thus, evidence is increasing that accumulation of fat in the liver in many instances cannot be regarded as a pathology or disease,

but rather as a physiologic response to increased caloric consumption [40]. Free fatty acids and cholesterol, especially when accumulated in mitochondria, are considered the ‘‘aggressive’’ lipids leading to TNF-α mediated liver damage and reactive oxygen species (ROS) [41,42]. These lipids could also be present in a nonsteatotic liver and act as early ‘‘inflammatory’’ hits leading to the wide spectrum of NAFLD pathologies. The concept of lipotoxicity and involved lipid species has been introduced and discussed in several excellent review articles [43,44].

Inflammation Preceding Steatosis

Simple hepatic steatosis, which is benign and nonprogressive in the majority of patients, and NASH may reflect different disease entities. Inflammation results in a stress response of hepatocytes, may lead to lipid accumulation, and therefore could precede steatosis in NASH. Patients with NASH may present without any or much steatosis, suggesting that inflammation could take place first [23]. Anti-TNF antibody treatment and metformin, an antidiabetic drug that inhibits hepatic TNF-α expression, improve steatosis in ob/ob mice [45,46]. Other proinflammatory mediators might also contribute to the development of steatosis because in some studies hepatic steatosis was not dependent on TNF-α [47]. In patients with severe alcoholic hepatitis, treatment with infliximab, an anti-TNF antibody, primarily improves hepatic steatosis [48]. Loss of Kupffer cells also leads to hepatic steatosis probably via decreased interleukin-10 (IL-10) release from Kupffer cells [49]. Other cell types might also promote hepatic steatosis because obesity leads to the hepatic recruitment of a myeloid cell population that further promotes hepatic lipid storage [50]. In all these situations, hepatic steatosis may be considered as ‘‘bystander phenomenon’’ subsequent to inflammatory attacks. Very diverse processes including toxic lipids, nutrients, and other gut-derived and adipose-derived signals may represent such inflammatory insults.

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 and soluble mediators, synthesized from immune cells and adipose tissue, are crucially involved in regulating insulin action and NAFLD occurrence [51,52]. The central role of IR in liver disease is further suggested by evidence that it is present also in non-obese, non-diabetic subjects with NAFLD [53]. Subjects with NAFLD and IR, present an impairment in muscle glucose uptake, an alteration in suppression

of hepatic endogenous glucose production induced by insulin [53,54], and a high lipolytic effect in adipose tissue resulting in an increased FFAs release [55]. The importance of visceral fat in the pathogenesis of hepatic IR and steatosis has been widely demonstrated in preclinical and clinical studies [56]. In particular, in an animal model of inherited leptin resistance using leptin-receptor-deficient Zucker (*fa/fa)* rat, the surgical resection of intra-abdominal fat depots reverses both hepatic IR and steatosis [57]. In humans, a clear relationship exists between hepatic IR and visceral fat leading to altered adipokine production and increased FFAs [58,59]. The enlargement of adipose tissue and in particular visceral fat has been associated with tissue inflammation characterized by a decreased release of insulin-sensitizing and anti-inflammatory cytokines and increased expression of pro-inflammatory molecules, which modify adipokine secretion [59]. Subjects with NAFLD exhibit decreased adiponectin levels [60], which are correlated negatively with hepatic triglyceride (TG) content. Interestingly, although the three-dimensional structure of adiponectin closely resembles that of TNF-α, these two proteins have completely opposite effects [61]. 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 [62]. 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 [63]. 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 (AMPK), p38 mitogen-activated protein kinase (p38 MAPK) and peroxisome proliferator-activated receptors α (PPAR-α) [64]. Other adipokines, such as leptin, visfatin and resistin, have also been reported to be involved in hepatic TG accumulation and inflammation. However, the role of these factors and their interplay is still to be elucidated [59]. 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 [65]. Altered lipid homeostasis in NAFLD negatively affects Toll-like receptor 4 (TLR4) complex assembly and sorting, leading alternative signalling pathways activation, such as NF-kB/AP1 (Nuclear factor-kB/ Activator protein 1), interferon regulatory factor 3 and promoting differential gene transcription. These differential pathways were similar not only in Kupffer cells and hepatic stellate cells, but also in other hepatic non-immune cell populations, including hepatocytes, biliary epithelial and endothelial cells [66,67]. Additional factors appear to interact with adiponectin to regulate hepatic triglyceride content. Among these, PPARs, belonging to the nuclear receptor superfamily, impact on multiple processes involved in lipid trafficking and

metabolism, and fuel partitioning [68]. In particular PPAR-α regulates mitochondrial and peroxisomal fatty acid β-oxidation pathways modulating many genes encoding 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 PPAR-α expression, in KO mice or in animal fed a methionine- choline deficient diet (MCD) or a high fat diet (HFD), results in hepatic steatosis [69,70]. In nutritional NAFLD models the administration of a potent PPAR agonist or probiotics results in an improvement of hepatic steatosis. These findings suggest that under conditions of increased hepatic fatty acid influx or decreased hepatic fatty acid efflux, PPAR-α activation prevents the accumulation of TG by increasing the rate of fatty acid catabolism [70,71].

A growing body of literature implicate PPARs in the pathogenesis and in the treatment of NAFLD, linking PPAR-α and PPAR-γ to NAFLD/NASH [72]. In fact, PPAR-γ are expressed at high levels in adipose tissue and play a role in increasing insulin sensitivity, as well as in promoting fatty acid uptake into adipocytes [73]. The net effect of PPAR-γ activation is the increase in adipocyte TG storage, reducing delivery of fatty acids to the liver. Moreover, PPAR-γ increases insulin sensitivity by up-regulating GLUT4, an insulin dependent glucose transporter in adipose tissue and striated muscle, and by inducing expression of the c-Cbl (named after Casitas B-lineage Lymphoma) associated protein, which is involved in insulin signalling [74]. Additionally, in mouse models of insulin resistance, PPAR-γ activation attenuated induction of suppressor of cytokine signalling 3 (SOCS3), which is involved in the development of insulin resistance [75]. PPAR-γ expression also might reduce hepatic inflammation by decreasing expression of proinflammatory cytokines, such as TNF-α [76]. Moreover, adiponectin is up-regulated by PPAR-γ, providing a connection between the two receptor isotypes [77]. The complexity and the chronology of pathophysiological events leading to development of NAFLD/NASH are not fully understood. The increased intrahepatic levels of FFAs provide a source of oxidative stress, which are in part responsible for the progression from steatosis to steatohepatitis and cirrhosis. FFAs may elicit hepatotoxicity by several mechanisms: direct cytotoxic effect [78], increased lysosomal permeability and TNF-α synthesis by hepatocytes [79].

Adipose Tissue-Derived Signals: The Adipose Tissue Factors Attack the Liver

Adipose tissue has appeared in the last decade as a highly active endocrine and immune organ with the capability of producing various mediators including adipocytokines and cytokines both in health and disease. The balance/imbalance of an adipose tissue ‘‘mediator cocktail’’ may profoundly affect not only the functions in the adipose tissue but also in other important target organs such as liver (**Figure 1.1**).

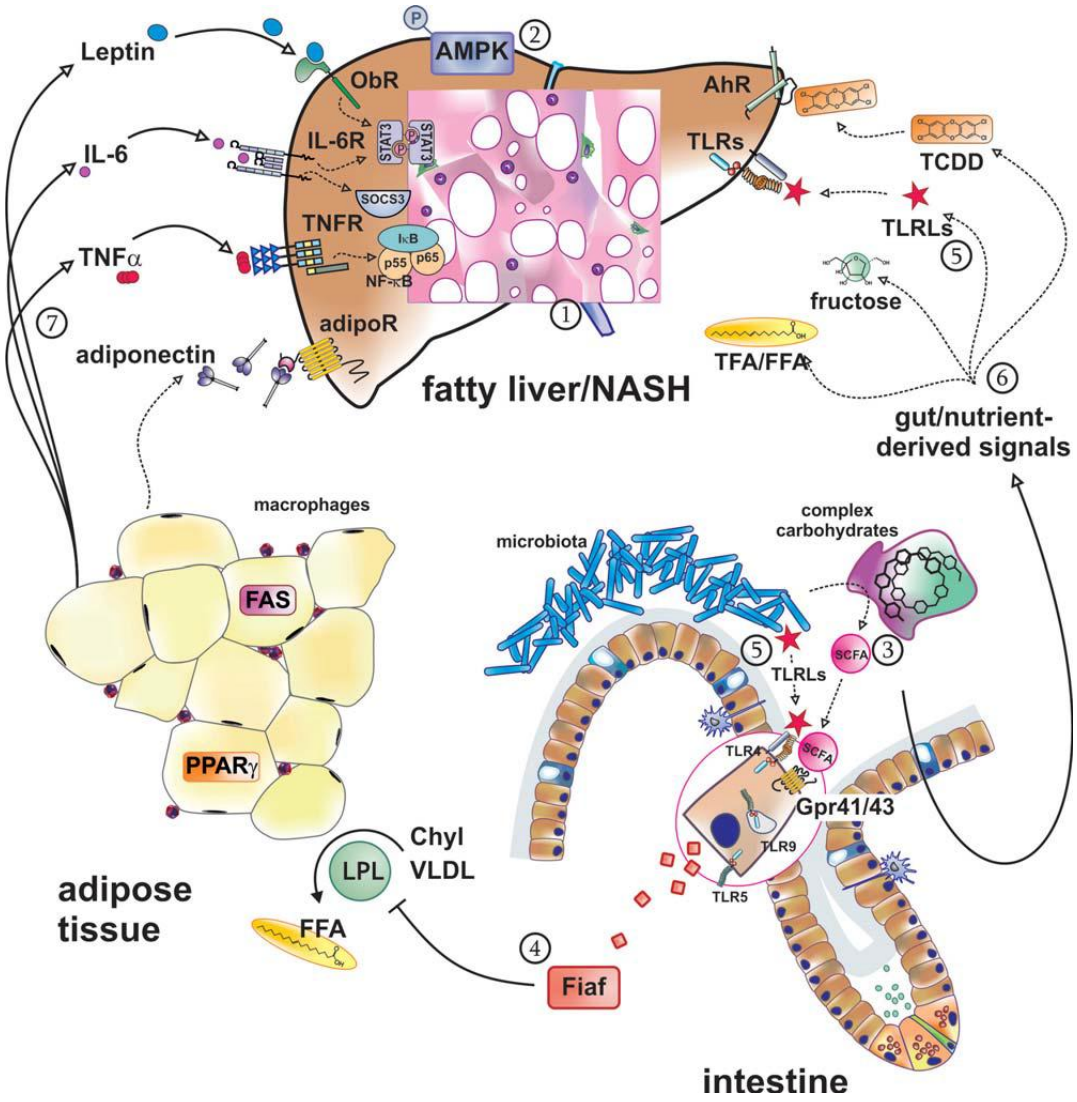


Fig. 1.1. The multiple parallel hits model. Lipotoxicity: (1) A liver loaded with lipids consisting primarily of trigylcerides might reflect a benign process because trigylcerides might exert mostly protective effects. Furthermore, hyperleptinemia leads to oxidation of hepatic lipids, thereby also protecting this organ from lipotoxicity. When the capacity of peripheral and central organs of detoxifying ‘‘aggressive lipids’’ fails, lipotoxic attack of the liver might begin. Inflammation may precede steatosis in NASH. Gut-derived signals: Many signals beyond endotoxin might affect hepatic steatosis and inflammation. Several pathways have been identified how the gut microbiota might influence host energy metabolism: (2) Absence of

the microbiota in germ-free mice correlates with increased activity of phosphorylated AMPK in the liver and the muscle (not shown). (3) Some of the breakdown products of polysaccharides are metabolized to SCFAs. SCFAs such as propionate and acetate are ligands for the G protein–coupled receptors Gpr41 and Gpr43. Shortage of SCFAs might allow the evolution of systemic inflammatory events. Such mechanisms elegantly combine diet, microbiota, and the epithelial cell as ‘‘nutrient sensor.’’ (4) The microbiota decreases epithelial expression of fasting-induced adipocyte factor (Fiaf), which functions as a circulating lipoprotein lipase (LPL) inhibitor and therefore is an important regulator of peripheral fat storage. (5) Several TLRs, such as TLR5 or TLR9, are not only able to affect microbiota but also to regulate metabolism, systemic inflammation, and insulin resistance, thus highlighting the role of the innate immune system in metabolic inflammation as observed in NASH. (6) Various nutrients such as trans fatty acids (TFAs), fructose or aryl hydrocarbon receptor (AhR) ligands such as 2,3,7,8-tetrachlorodibenzodioxin (TCDD) may directly lead to steatosis/liver inflammation. Adipose tissue–derived signals: Signals derived from the adipose tissue beyond toxic lipids might play a central role in NAFLD/NASH. (7) Here, adipocytokines such as adiponectin and leptin, certain proinflammatory cytokines such as TNF-α or IL-6, and others (the death receptor Fas, PPAR-γ) are of key relevance. The cytokine/adipocytokine milieu might be critical because ob/ob-adiponectin tg mice, although becoming severely obese, are not insulin-resistant. This suggests that in the hierarchy of processes soluble mediators play the central role. Adipose-derived mediators might indeed affect target organs such as the liver, because JNK1 adipose-deficient mice are protected from diet-induced obesity, and experiments have demonstrated that this effect is mediated mainly by IL-6 (a cytokine), which is of key importance in human obesity.
Image from Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. Hepatology. 2010 Nov;52(5):1836-46. doi: 10.1002/hep.24001.

Adiponectin: Prototypic Adipocytokine in Health and Disease

Adiponectin is an anti-inflammatory adipocytokine that signals through two receptors [80,81]. Obesity is associated with hypoadiponectinemia, and adiponectin levels increase after weight loss [80]. Adiponectin induces extracellular Ca^{2+} influx by adiponectin receptor 1, which is necessary for activation of AMPK and Sirtuin 1 (Sirt1) [82]. Hepatocyte-specific deletion of Sirt1 leads not only to hepatic steatosis but also to ER stress and liver inflammation [83]. Genetically obese leptin-deficient ob/ob mice exhibit a reversal of the diabetic phenotype with normalization of glucose and insulin levels upon transgenic overexpression of the full-length isoform of adiponectin, despite retaining the obese phenotype [84]. Therefore, also in humans, a sufficient production of adiponectin might play a central role in susteining homeostasis and establishing a balance when local and systemic/ liver inflammation is compromised [85].

IL-6 and TNF-α: Key (Adipo)cytokines

Expression of IL-6 and TNF-α, two important proinflammatory cytokines, is profoundly increased in human fat cells from obese subjects and patients with insulin resistance [86]. IL-6 serum levels are elevated in obese patients and weight loss results in their reduction [87,88]. Enhanced TNF-α expression in adipose tissue of obese subjects decreases following weight loss [89]. Insulin resistance is an important feature of NAFLD and it is caused by several factors,

including soluble mediators derived from immune cells and/or adipose tissue [90]. Insulin resistance may augment inflammation in NASH because patients with type 2 diabetes mellitus are often worsen in terms of histopathological changes such as ballooning, apoptosis, and lobular and/or portal inflammation [23]. Serine phosphorylation of insulin receptor substrate by inflammatory signal transducers such as c-jun N-terminal protein kinase 1 (JNK1) or inhibitor of nuclear factor-kB kinase-β (IKK β) is considered one of the key aspects that impairs insulin signalling (**Figure 1.2**). Sabio et al. reported that JNK1 signaling specifically in adipose tissue consequent to a high-fat diet causes hyperinsulinemia, hepatic steatosis, and hepatic insulin resistance [91]. Importantly, this distal effect of adipose tissue on the liver was mediated via increased JNK1-dependent IL-6 secretion from adipocytes, proving that adipose tissue–derived IL-6 regulates distal metabolic effects in the liver. In this and in other models, a high-fat diet, that can be considered ‘‘an inflammatory diet’’, is a prerequisite and a key player in the etiophatogenesis including hepatic steatosis and other related pathologies.

Moschen et al., recently demonstrated that such a mechanism as suggested by Sabio et al. might also be operative in human obesity [92]. In this study, IL-6 expression has been more than 100-fold higher in adipose tissue (subcutaneous and visceral) compared to its liver expression, suggesting that in severe obesity, the adipose tissue is indeed the major source of IL-6. Weight loss resulted in a dramatic decrease of IL-6 and TNF-α expression with subsequent reduced expression of SOCS3 expression and improved insulin sensitivity, evidencing the hepatic consequences of adipose tissue alterations. So, the liver can be considered a key target organ for adipose tissue–derived IL-6 and TNF-α, because continuous IL-6/ TNF-α exposure affects hepatic insulin resistance, e.g., via up-regulation of SOCS3 [93].

Interestinly, enhanced expression of proinflammatory cytokines in adipose tissue was observed, although liver inflammation was still absent, suggesting that adipose tissue inflammation could precede liver inflammation [92]. Many human studies suggest that the amount of visceral fat directly correlates with degree of hepatic steatosis and inflammation. Hepatic inflammation and fibrosis correlate with the amount of visceral fat [94]. Abdominal fat has been shown to be a major factor leading to increased serum alanine aminotransferase levels, which might reflect more advanced disease such as NASH [95]. This important clinical study further supports the important association between adipose tissue and liver disease. Besides certain adipocytokines/immune mediators, the cellular infiltrate in the adipose tissue must be also considered. In fact, the reduction of adipose macrophages (CD11c+ cells) improves insulin sensitivity and decreases inflammation [96]. Importantly, adiponectin and PPAR-γ promote adipose tissue macrophage polarization toward

an alternative/anti-inflammatory phenotype [97,98]. Other studies [99] present evidence that adipose tissue inflammation is a common event in morbid obesity, and this tissue could be considered-the major cytokine source in obesity.

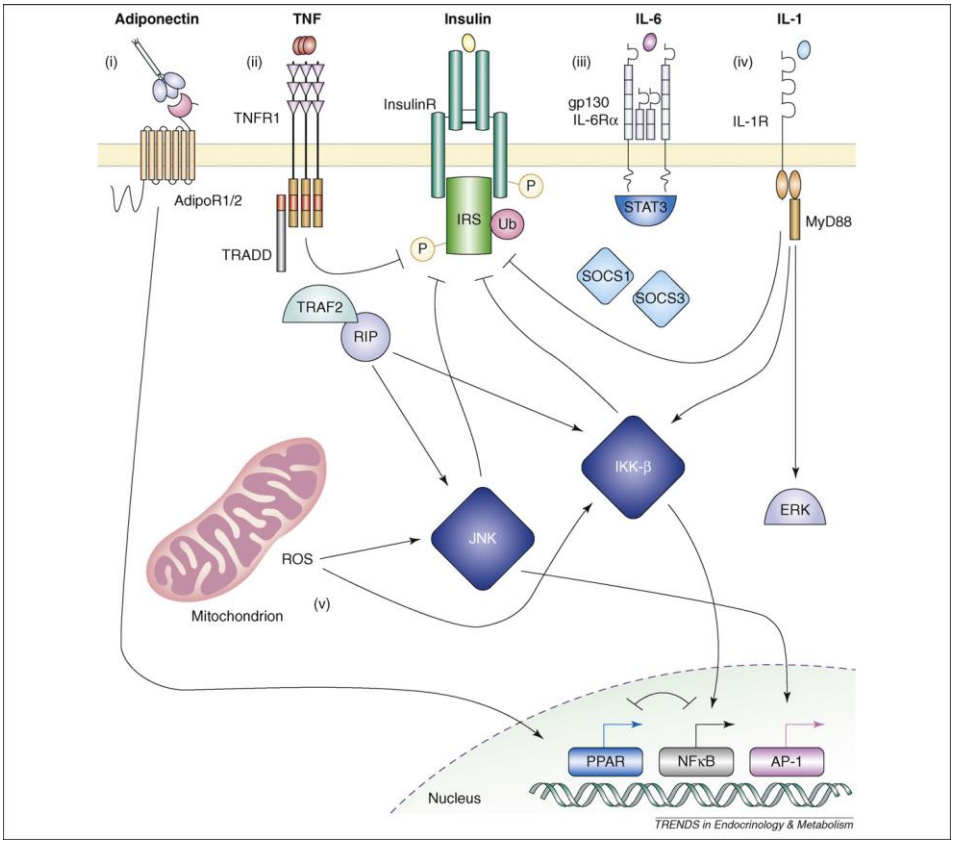


Fig. 1.2. Regulation of insulin resistance and involved pathways. Several inflammatory pathways involved in the regulation of IR have been identified. (i) adiponectin, an important insulin-sensitizing adipocytokine that signals via adiponectin receptors. (ii) TNF-α was among the first mediators to be defined as an important factor linking inflammation, obesity and IR. Engagement of TNFR by TNF-α induces inhibitory phosphorylation of serine residues on IRS1 and activates IKKβ and NF-kB pathways and JNK pathways, two major intracellular regulators of IR. (iii) IL-6 is another pro-inflammatory cytokine involved in IR development. This cytokine activates SOCS1 and SOCS3 that initiate ubiquitin-mediated degradation of IRS. (iv) IL-1 has been shown to reduce IRS1 expression via ERK1/2 and can also activate the IKKβ and NF-kB pathway. (v) Oxidative stress also regulates inflammation-associated IR.

Abbreviations: AP-1, activator protein 1; ERK, extracellular receptor kinase; IKKβ, inhibitor of kappa B kinase β; IL, interleukin; insulinR, insulin receptor; IR, insulin resistance; JNK, c-Jun N-terminal kinase; NF-kB, nuclear factor kappa B; PPAR, peroxisome proliferator activated receptor; RIP, receptor interacting protein; ROS, reactive oxygen species; SOCS, suppressor-of-cytokine-signaling; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor-α; TRADD, TNF receptor-1 associated death domain protein; TRAF2, TNF receptor-associated factor 2; Ub, ubiquitin mediated. *Image from Tilg H, Moschen AR. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol Metab. 2008 Dec;19(10):371-9. doi: 10.1016/j.tem.2008.08.005. Epub 2008 Oct 17. Review.*

Liver-gut axis

Due to its anatomical links to the gut, the liver is the major filter organ and a first line defence 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, playing a pivotal role for the protection in systemic bacterial infection [100]. Toll-like receptors (TLRs) recognise 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 gut hosts more than 99% of the bacterial mass in the body, intestinal microbiota is the principal source of bacterial-derived PAMPs 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 (DAMPs), act as signal of the presence of necrosis and subsequently trigger inflammation [66,101]. The healthy liver contains low mRNA levels of TLRs (TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10) and signalling molecules (i.e. CD14 cluster of differentiation 14, MD-2 or Lymphocyte antigen 96 and MyD88 myeloid differentiation primary response 88) in comparison to other organs, suggesting that the low expression of TLR signalling molecules may contribute to the high tolerance of the liver to TLR ligands deriving from the intestinal microbiota [67,102]. In chronic liver diseases, for instance cirrhosis, structural changes of the intestinal mucosa (e.g. loss of tight junctions TJs, widening of intercellular spaces, vascular congestion, and defects in the mucosal immune system) promote the loss of barrier function and allow translocation of bacteria and bacterial PAMPs [102]. Many proinflammatory effects of PAMPs are a consequence of TLR-induced secretion of inflammatory mediators, such as TNF-α and interleukin (IL)-1β as demonstrated both *in vitro* and *in vivo* [103].

Gut-liver axis suggests a tight linkage between the health of intestinal tract and that of liver. In fact, there is growing evidence of how gut microflora alteration or dysbiosis may affect liver pathology. Altered intestinal bacterial flora as a result 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, were proposed. Normally, intestinal anaerobic bacteria outnumber aerobic bacteria, the latter are responsible of bacterial translocation. Thus, anaerobic bacteria, suppressing the

colonisation 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 [104].

Endotoxin and Its Role in Obesity

Endotoxin (lipopolysaccharide -LPS), a key constituent of many bacteria present in our microbiota, plays a central role in innate immune responses and has been considered the so-called ‘‘second hit’’ in previous NASH models [28]. Manipulation at the gut surface, including dietary ingredients, may affect LPS metabolism and result in increased circulating plasma levels. It has been demonstrated that intake of a high-fat or a high-carbohydrate diet in humans over only 3 days leads to an increase in circulating LPS concentrations [105]. Endotoxemia, however, might not only lead to systemic inflammation but might also worsen obesity itself [106]. When endotoxemia was induced for 4 weeks in lean mice, liver and adipose tissue weight gain were increased similarly as after a high-fat diet. This weight gain was paralleled by hepatic insulin resistance, and could be prevented by antibiotic therapy. Patients with NAFLD demonstrate increased gut permeability, which importantly has been associated with the severity of liver steatosis but not with the degree of inflammation (NASH) [107]. This study therefore suggests that gut derived factors/signals such as endotoxin might also affect accumulation of hepatic fat.

Intestinal Epithelium: Linking Nutrients to Metabolic Diseases

To date it is be recognized that our microbiota might influence systemic immune responses. At least in part this effect might take place via the bacterial capacity to digest dietary fiber resulting in the production of short-chain fatty acids (SCFA). SCFAs have anti-inflammatory functions in various models of colitis and human ulcerative colitis probably via interaction with its receptor, the G protein–coupled receptor 43 (Gpr43) [108].

Gpr43-/- mice show systemic inflammation in various tissues [109], similar to germ-free wild-type mice devoid of bacterial fermenting capacity and hence with almost absent SCFAs in the gut. Various other pathways (i.e., *Fiaf*; Gpr41) have been characterized interfering with

metabolism/adiposity, highlighting how the intestinal microbiota and its products might directly regulate host gene expression and affect systemic inflammation [110,111]. These pathways involve the intestinal epithelium as ‘‘sensor’’ of the microbiota, implicating a major role for the intestinal epithelium in determining systemic metabolic functions. Interference with our microbiota via probiotics or prebiotics or SCFAs might therefore be beneficial and improve systemic inflammation/metabolic function. So far, only a few animal studies have been performed [112,113].

Toll-Like Receptors and Role of Innate Immunity in Obesity-Related Inflammation

Toll-like receptors (TLRs), also expressed on the gut epithelium, can respond to nutritional lipids such as free fatty acids and might thereby have a role in the pathogenesis of obesity-associated inflammation/insulin resistance [114]. The recognition of fatty acids by TLR4 can induce the production of proinflammatory cytokines in macrophages and epithelial cells [115]. TLR-4–deficient mice are protected from high-fat diet-induced inflammation and insulin resistance [116]. It is, however, not universally accepted that saturated free fatty acids are ligands for certain TLRs because it has been demonstrated that saturated fatty acids might not directly stimulate TLR-dependent signalling [117]. Therefore, the *in vivo* effects observed in the above discussed study [115] could also be accounted by gut-derived endotoxin or by endotoxin contamination of the lipids employed.

Other TLRs may also be involved in obesity-related inflammation. TLR9 promotes steatohepatitis because TLR9-deficient mice are protected from liver inflammation [118]. The importance of the gut as ‘‘metabolic organ’’ has been convincingly demonstrated by a recent report indicating that mice deficient in TLR5 develop all features of metabolic syndrome including hyperphagia, obesity, insulin resistance, pancreatic inflammation, and hepatic steatosis [119]. TLR5 deficiency affected the composition of the gut microbiota and, remarkably, transfer of the microbiota from TLR5 -/- mice to healthy mice resulted in transfer of disease. There are two major implications of this work: (1) the innate immune system plays a critical role in the development of the metabolic syndrome and (2) transfer of the gut microbiota to wild-type germ-free mice results in several features of *de novo* disease (i.e., metabolic syndrome), again supporting a major role for our microbiota in metabolic inflammation.

Treatment for NAFLD/NASH

Current therapies for NAFLD and NASH are focused on the various pathways or process thought to be central in the pathogenesis of this disease. Treatment regimens targeting insulin resistance, oxidative stress, diabetes, hyperlipidemia, obesity, and hepatic fibrosis all warrant critical appraisal. Multiple modalities include diet, exercise, surgical interventions, and finally pharmacotherapy require evaluation to determine the most effective treatment algorithms. Although one panacea has not been found and is unlikely to exist, a multimodality treatment regimen might prove effective (**Figure 1.3**).

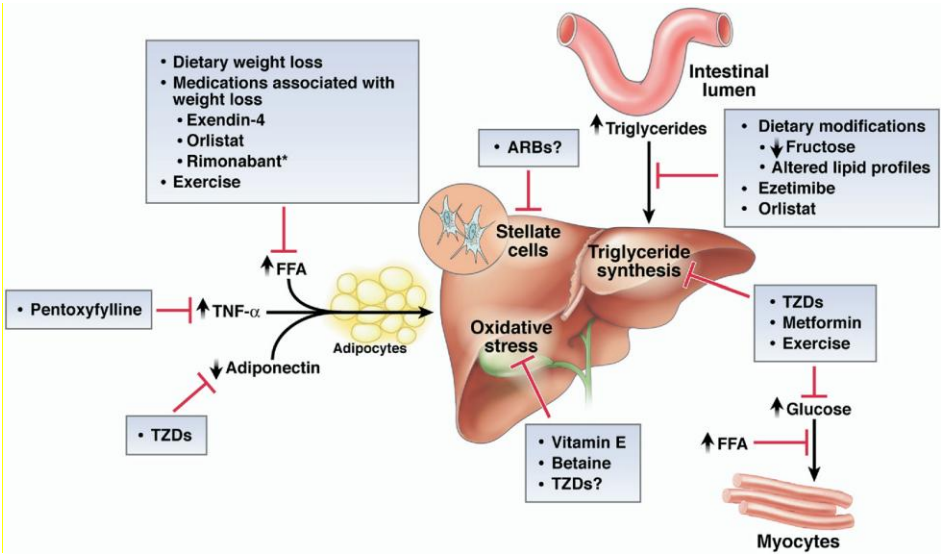


Fig. 1.3. Potential pathophysiologic effects of therapies that are under investigation. The development of hepatic steatosis and subsequent steatohepatitis is multifaceted. Several therapeutic modalities under investigation such as the TZDs (Thiazolidinediones), diet, and exercise, and possibly rimonabant, may have pleiotrophic effects in improving NAFLD.

*Endocannabinoid receptors have been identified in multiple organs involved in energy homeostasis to include adipocytes, liver, and possibly skeletal muscle and the pancreas. *Image from: Torres DM, Harrison SA. Diagnosis and therapy of nonalcoholic steatohepatitisGastroenterology. 2008 May;134(6):1682-98. doi: 10.1053/j.gastro.2008.02.077*.

CHAPTER 2: ULCERATIVE COLITIS

Ulcerative colitis (UC) was first described in the mid-1800s, whereas Crohn's disease (CD) was first reported later, in 1932, as "regional ileitis." Because Crohn's disease can involve the colon and shares clinical manifestations with ulcerative colitis, these entities have often been conflated and diagnosed as inflammatory bowel disease (IBD), although they are clearly distinct pathophysiological entities. Ulcerative colitis is the most common form of inflammatory bowel disease worldwide. In contrast to Crohn's disease, ulcerative colitis is a disease of the mucosa that is less prone to complications and can be cured by means of colectomy, and in many patients, its course is mild [120]. The literature on the pathogenesis and treatment of so-called IBD has tended to focus on Crohn's disease [121,122], and few articles expressly discuss ulcerative colitis [123,124]. Ulcerative colitis and Crohn's disease are disorders of modern society, and their frequency in developed countries has been increasing since the mid-20th century. When IBD is identified in a new population, ulcerative colitis invariably precedes Crohn's disease and has a higher incidence. Among children, however, ulcerative colitis is less prevalent than Crohn's disease [125]. The highest incidence and prevalence of IBD are seen in the populations of Northern Europe and North America and the lowest in continental Asia, where ulcerative colitis is by far the most common form of inflammatory bowel disease [126]. A westernized environment and lifestyle is linked to the appearance of IBD, which is associated with smoking, diets high in fat and sugar, medication use, stress, and high socio-economic status [127]. IBD has also been associated with appendectomy [127]. Of these factors, only cigarette smoking and appendectomy are reproducibly linked to ulcerative colitis.

Symptoms, Clinical Course, and Assessment of Disease Activity

Bloody diarrhea with or without mucus is the hallmark of ulcerative colitis. The onset is typically gradual, often followed by periods of spontaneous remission and subsequent relapses. Active disease is manifested as mucosal inflammation commencing in the rectum (proctitis) and in some cases spreading to the rest of the colon (**Fig. 2.1A**). Although proctitis is frequently associated with fecal urgency and the passage of fresh blood, constipation may paradoxically occur.

Proctosigmoiditis, leftsided colitis, extensive colitis, or pancolitis (**Fig. 2.1B**) may lead to diarrhea, frequent evacuations of blood and mucus, urgency or tenesmus, abdominal pain, fever, malaise, and weight loss, depending on the extent and severity of the disease. The prognosis for patients with ulcerative colitis is generally good during the first decade after diagnosis, with a low rate of colectomy; over time, remission occurs in most patients [120]. Assessment of the clinical activity of ulcerative colitis helps the clinician choose, diagnostic tests and make therapeutic decisions.

A **B**

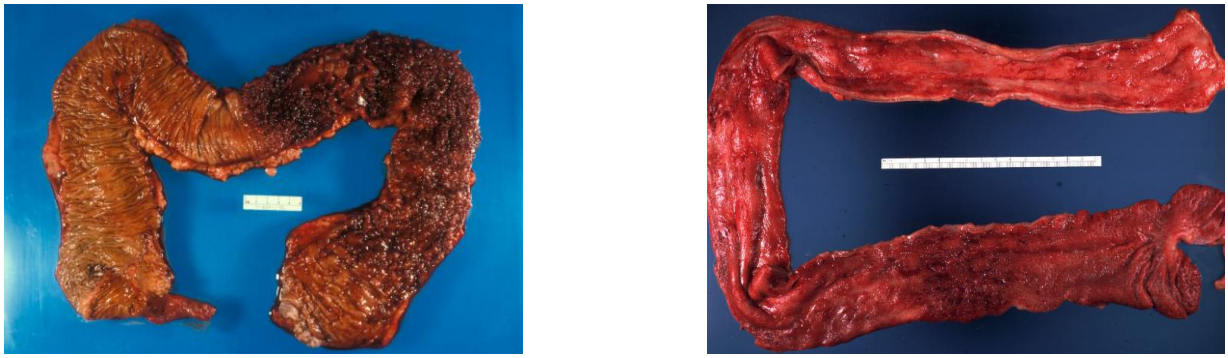


Fig. 2.1. Gross morphological appearance of typical UC specimens. **A**. Severe inflammation of the rectum, sigmoid, splenic flexure and part of the transverse colon, where it stops abruptly and transitions to normal mucosa; **B**. Severe pancolitis; Pictures taken from: *Danese S, Fiocchi C. Ulcerative colitis. N Engl J Med. 2011 Nov 3;365(18):1713-25. Doi 10.1056/NEJMra1102942.Review.*

Diagnosis

An accurate diagnosis of ulcerative colitis involves defining the extent and severity of inflammation, and this information provides the basis for selecting the most appropriate treatment and for predicting the patient's prognosis. Both endoscopy and biopsy are required to determine specific histologic characteristics; radiologic and ultrasonographic examinations are not critical but may be useful [128]. All these investigations aid in differentiating ulcerative colitis from other conditions that have similar sympthoms. Colonoscopy shows a uniformly inflamed mucosa that starts at the anorectal verge and extends proximally, with an abrupt or a gradual transition from affected to normal mucosa. In mild ulcerative colitis, the mucosa has a granular, erythematous appearance, with friability and loss of the vascular pattern. In moderate disease, erosions or microulcerations are evident, whereas in severe ulcerative colitis, shallow ulcerations with

spontaneous bleeding are generally seen (**Fig. 2.2A and 2.2B**). Colonoscopy helps to differentiate ulcerative colitis from Crohn's disease, which is typically characterized by rectal sparing, aphthous ulcers, skip lesions (areas of inflammation alternating with normal mucosa), a cobblestone pattern, and longitudinal, irregular ulcers. In patients with cycles of inflammation and healing and in those with chronic, unremitting inflammation, colonoscopy may reveal pseudopolyps or mucosal bridging. Although there is no clear evidence that surveillance prolongs survival [129], biopsy specimens should be taken from all colonic segments, regardless of whether they are inflamed, with a particular focus on irregular mucosa, polypoid lesions, and any raised, dysplasia-associated lesion or mass [130].

A **B**

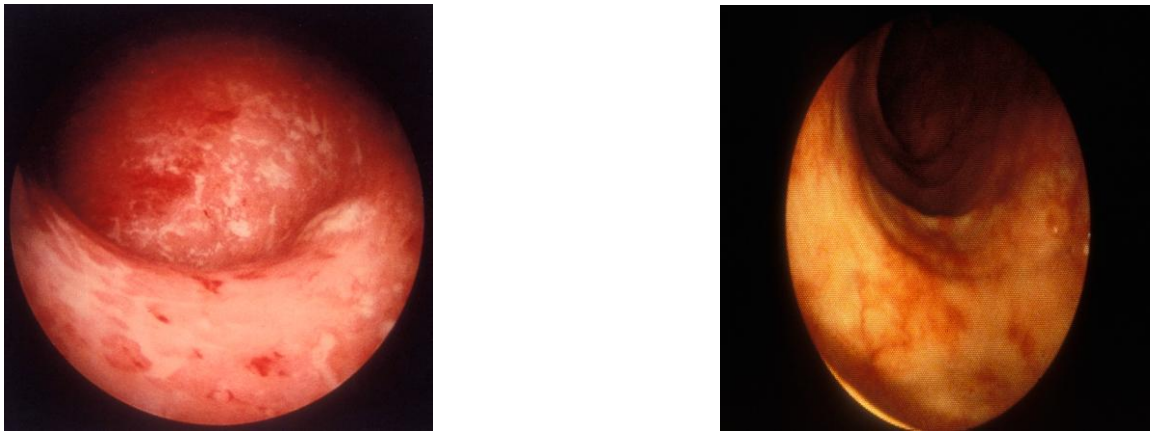


Fig. 2.2. Endoscopic appearance of UC. **A**. Severe active colitis with ulcerations and spontaneous bleeding; **B**. Chronic active colitis. Pictures taken from: *Danese S, Fiocchi C. Ulcerative colitis. N Engl J Med. 2011 Nov 3;365(18):1713-25. Doi 10.1056/NEJMra1102942.Review*.

Histologic Evaluation

In ulcerative colitis, inflammation is characteristically restricted to the mucosal layer, with infiltrates varying in density and composition during active disease or stages of remission (**Fig. 2.3A and 2.3B**). Infiltrates consist primarily of lymphocytes, plasma cells, and granulocytes; the last are being particularly prominent during acute flare-ups and accumulate in crypt abscesses [131] (**Fig. 2.3C**). Other typical features include goblet- cell depletion, distorted crypt architecture, diminished crypt density, and ulcerations. However, epithelioid granulomas, which are typical of Crohn's disease, are not present. Looking for epithelial dysplasia is critical, given the risk of cancer in patients with long-standing ulcerative colitis; however, dysplasia can occur at any stage without

indicating malignant transformation There are no exact criteria for the diagnosis of ulcerative colitis, but in most cases, the presence of two or three of the aforementioned histologic features will suffice [132]. The severity of inflammation on histologic examination and the severity of disease on endoscopic examination may not coincide; for instance, histological findings (**Figure 2.3**) may indicate severe disease even in a patient with endoscopically quiescent disease.

A **B** **C**

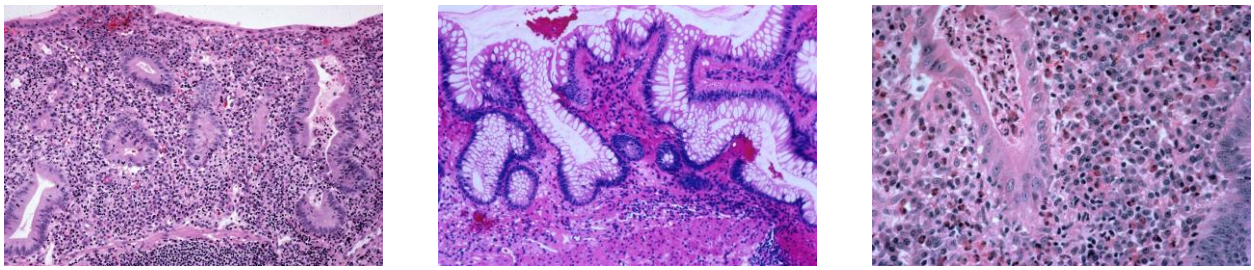


Fig. 2.3. Histologic appearance of typical UC mucosal tissue. **A**. Chronic active colitis with dense and diffuse inflammatory infiltrates; **B**. Chronic quiescent colitis with crypt distortion; **C**. Neutrophil accumulation in a crypt (crypt abscess) in severe colitis with massive leukocyte infiltration of the *lamina propria*. Pictures taken from: *Danese S, Fiocchi C. Ulcerative colitis. N Engl J Med. 2011 Nov 3;365(18):1713-25. Doi 10.1056/NEJMra1102942.Review*.

Genetic Features

The discovery that NOD2 (Nucleotide-binding oligomerization domain-containing protein 2) variants are associated with susceptibility to Crohn's disease opened a new era in the study of the genetic basis of inflammatory bowel disease [133,134]. In studies of twins, there is stronger concordance with Crohn's disease than with ulcerative colitis, and the identification of a large number of susceptibility loci for Crohn's disease in early genomewide association studies suggested that genetic influences play a greater role in Crohn's disease than in ulcerative colitis [135]. A meta-analysis of six such studies recently confirmed the presence of 47 loci associated with ulcerative colitis, of which 19 are specific for ulcerative colitis and 28 are shared with Crohn's disease [136]. Several pathways potentially associated with ulcerative colitis were identified in the meta-analysis and in individual studies based on validated loci or chromosomal regions [137]. Risk loci for ECM1, HNF4A, CDH1, and LAMB1 implicate dysfunction of the epithelial barrier; an association with DAP suggests a link to apoptosis and autophagy; and associations with PRDM1, IRF5, and NKX2-3 suggest defects in transcriptional regulation. In addition, multiple genes in the interleukin-23 signaling pathway overlap in ulcerative colitis and Crohn's disease (e.g., IL-23R,

JAK2, STAT3, IL-12B, and PTPN2). Several risk loci linked to other immune system–mediated diseases are associated with ulcerative colitis, particularly HLA-DR and genes involved in helper T-cell types 1 and 17 (Th1 and Th17) differentiation, such as IL-10, IL-17R, IL-23R, and Interferon gamma (IFN-γ). Altogether, genetic studies indicate that both specific and non specific gene variants are associated with ulcerative colitis, and the two forms of inflammatory bowel disease share disease pathways. Ulcerative colitis appears to be as genetically heterogeneous as Crohn's disease, but given the large number of implicated genes and the small additive effect of each, genetic screening is not currently indicated to assess the risk of ulcerative colitis.

Microbiologic Features

The gut immune system is generally tolerant of this microbial load, and a breakdown in tolerance is postulated to be central to the pathogenesis of IBD [138]. Although loss of tolerance to gut microbiota is demonstrable in animal models of IBD, there are only limited evidence for this finding in patients with Crohn's disease and none in those with ulcerative colitis. It has also been postulated that alterations in the composition of the gut microbiota, defects in mucosal immunity, or the two factors combined could lead to ulcerative colitis; however, supportive evidences are sparse. A key issue is the characterization of the gut microbiota in the normal intestine and in the intestine in patients with IBD. This issue awaits answers from the Human Microbiome Project, which aims to define the composition of the intestinal microbiota in conditions of health and disease [139]. There is a consensus that the density of microbiota is greater in patients with ulcerative colitis or Crohn's disease than in healthy control subjects, but whether there are reproducible, disease-specific alterations is unclear [140]. The fact that antibiotic therapy has no clinical effect on ulcerative colitis argues against an important role of bacteria in this disease, whereas antibiotics do provide some benefit in luminal Crohn's disease. Although serum antibacterial antibodies are present in patients with ulcerative colitis, they are much more common and are found in higher titers in patients with Crohn's disease. Furthermore, the range of antibodies against bacterial antigens (anti-I2, anti-OmpC, and anti-CBir1 antibodies) and fungal antigens (anti–*Saccharomyces cerevisiae* antibodies [ASCA]) is broader in Crohn's disease, whereas the only ulcerative colitis–associated antibody is perinuclear antineutrophil cytoplasmic antibody (pANCA), which recognizes nuclear antigens that may cross-react with bacterial antigens [141].

Mucosal Immune Response

Intestinal homeostasis requires a controlled innate immune response to the microbiota, which is recognized by toll-like receptors and NOD receptors on epithelial and immune cells [142]. This recognition process contributes to tolerance, but when the process is dysregulated, inflammation ensues. At present, there is no clear evidence of specific, innate immune defects in ulcerative colitis; an increased expression of TLR2 and TLR4 by colonocytes [143] is probably secondary to inflammation. In contrast, in Crohn's disease, abnormalities of innate immunity are linked to variants of the NOD2, ATG16L1, and IRGM genes, the products of which normally mediate microbial recognition [144,145]. The production of proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and tumor necrosis factor–like ligand 1 (TL1A), is universally increased in patients with IBD but it does not allow to discriminate between ulcerative colitis and Crohn's disease. Abnormalities in humoral and cellular adaptive immunity occur in ulcerative colitis. Elevated IgM, IgA, and IgG levels are common in inflammatory bowel disease, but there is a disproportionate increase in IgG1 antibodies in ulcerative colitis [146]. Abnormalities of adaptive immunity that differentiate ulcerative colitis from Crohn's disease are defined by mucosal CD4+ T cells, which were initially divided into two lineages: Th1 and type 2 helper T cells (Th2). Crohn's disease is a Th1-like condition, on the basis of evidence of increased production of IFN-γ [147]. In contrast, ulcerative colitis represents an atypical Th2 response, as indicated by the presence of non classical natural killer T cells in the colon that secrete abundant IL-13, which mediates epithelial- cell cytotoxicity, apoptosis, and epithelial barrier dysfunction [148,149]. IL-5–producing Th2-polarized T cells are also present in ulcerative colitis. The balance between Th1 and Th2 has been used to differentiate between ulcerative colitis and Crohn's disease. However, additional helper-cell lineages have recently been delineated, including Th17 cells that produce the proinflammatory cytokine IL-17, the levels of which are increased in the mucosa of patients with IBD [150].

Epithelial Cells and Autoimmunity

Because inflammation in ulcerative colitis typically does not extend into the small intestine and occurs in proximity to the epithelium, colonocytes are implicated in the pathogenesis of this disease. It has been proposed that the epithelium is diffusely abnormal, irrespective of inflammation [151]. Other reported abnormalities in ulcerative colitis include an epithelial-barrier defect and impaired expression of PPAR-γ, a nuclear receptor that regulates inflammatory genes [152]. In both

ulcerative colitis and Crohn's disease, epithelial cells have a decreased ability to activate suppressor CD8+ T cells, but this abnormality is probably secondary to other immune events [153]. Variants of the XPB1 gene, the product of which is a component of the stress response of the endoplasmic reticulum in epithelial cells, have been linked to IBD, reinforcing the idea that colonocytes are involved in its pathogenesis [154]. Autoimmunity may play a role in ulcerative colitis. In addition to pANCA, this disease is characterized by circulating IgG1 antibodies against a colonic epithelial antigen that is shared with the skin, eye, joints, and biliary epithelium [155]; since these are the sites of extraintestinal manifestations in ulcerative colitis, it is possible that crossreacting antibodies against the colon cause organspecific damage. Tropomyosin 5, a structural protein, is the putative target autoantigen of the IgG1 antibodies [156], but evidence of classical antibody- mediated autoimmunity in ulcerative colitis is still lacking. **Figure 2.4** summarizes our current understanding of the pathogenesis of ulcerative colitis.

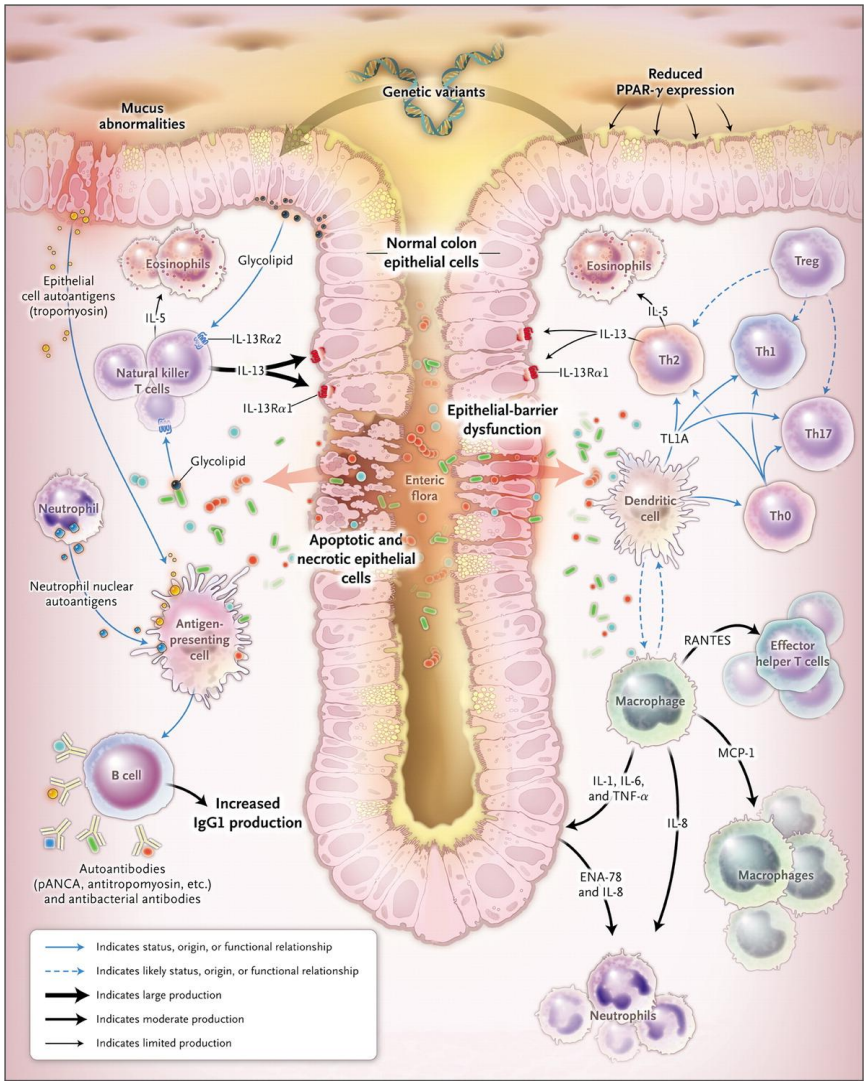


Fig. 2.4. Current concepts concerning the pathogenesis of ulcerative colitis.Glycolipids from epithelial cells, bacteria, or both induce the up-regulation of interleukin-13 receptor α2 (IL-13 α2) on mucosal natural killer T cells; autocrine interleukin-13 (IL-13) activates these cells, which expand in number and create a positive feedback loop that enhances interleukin-13–mediated natural killer T-cell cytotoxicity, causing epithelial-barrier dysfunction. This leads to enhanced absorption of bacterial products and the generation of antibacterial antibodies; damage to epithelial cells induces the production of anti-tropomyosin antibodies by B cells, while nuclear proteins from neutrophils induce the production of pANCA. In addition to type 1 and type 17 helper T cells (Th1 and Th17), an increased number of type 2 helper T cells (Th2) produce interleukin-13, which induces epithelial-barrier dysfunction, resulting in increased permeability, and interleukin-5 (IL-5), which may contribute to eosinophil recruitment and activation. Increased absorption of bacterial products stimulates dendritic cells and macrophages, resulting in the production of proinflammatory cytokines and chemokines. Interleukin-1β–activated epithelial cells secrete epithelial neutrophil-activating peptide 78 (ENA-78) and interleukin-8, which recruit neutrophils, as well as monocyte chemoattractant protein 1 (MCP-1), which attracts and activates macrophages, and RANTES (regulated upon activation, normal T cell expressed and secreted), which attracts and recruits effector helper T cells. Genetic variants associated with ulcerative colitis, reduced expression of PPAR-γ by colonocytes, mucus abnormalities, and abnormalities of regulatory T cells (Treg) may also contribute to selective autoimmune and immune-mediated events in the pathogenesis of ulcerative colitis. IL-1 denotes interleukin-1, IL-6 interleukin-6, TL1A tumor necrosis factor–like ligand 1, and TNF-α tumor necrosis factor α. *Image from: Danese S, Fiocchi C. Ulcerative colitis. N Engl J Med. 2011 Nov 3;365(18):1713-25. Doi 10.1056/NEJMra1102942.Review*.

Pharmacological Therapy

According to current consensus-based guidelines, the choice of drug treatment for patients with ulcerative colitis should take into consideration the level of clinical activity (mild, moderate, or severe) combined with the extent of disease (proctitis, left-sided disease, extensive disease, or pancolitis), the course of the disease during follow-up, and patient' s preferences [157,158].

Drugs for Remission Induction

Sulfasalazine and 5-aminosalicylates (mesalamine, olsalazine, and balsalazide), given orally, rectally (by means of suppository or enema), or both, represent first-line treatment for ulcerative colitis, with an expected remission rate of about 50%. Mild-to-moderate proctitis can be treated with mesalamine suppositories (1 g per day) or enemas (2 to 4 g per day); clinical remission occurs in most patients within 2 weeks, with repeated treatments as needed. If it fails, 5-aminosalicylate enemas (2 to 4 g per day) or glucocorticoid enemas (hydrocortisone at a dose of 100 mg per day, or new preparations such as budesonide or beclomethasone) are the next step [159]. Patients who do not have a response to rectally administered agents may be given oral glucocorticoids (up to 40 mg of prednisone or its equivalent). Patients with mild-to-moderate ulcerative colitis that are refractory to rectal therapies and to oral 5-aminosalicylate are candidates for oral glucocorticoids or immunosuppressive agents (azathioprine or 6-mercaptopurine); to whom do not have a response to maximal doses of 5-aminosalicylate or oral glucocorticoids should be given intravenous glucocorticoids [157].

For patients who continue to require glucocorticoid therapy and for those who do not have a response to it, a good therapeutic option appears to be infliximab, a monoclonal antibody against TNF-α, administered at a dose of 5 mg per kilogram of body weight at 0, 2, and 6 weeks [160]. Infliximab in combination with azathioprine (2.5 mg per kilogram) were reported to be more active than infliximab or azathioprine monotherapy for inducing glucocorticoid free remission in patients with moderate-to-severe ulcerative colitis. Many specialists suggest that patients with extensive, severe disease receive a 5-day to 7-day course of intravenous glucocorticoids [157]; if the disease is unresponsive, then intravenous cyclosporine (2 mg per kilogram) or infliximab is usually the next step. Although cyclosporine can be effective, it generally delays rather than prevents subsequent colectomy [161]; furthermore, infliximab is increasingly used as an alternative treatment for

patients with refractory disease, given its effectiveness and better short-term safety profile as compared with other therapies [162].

Drugs for Remission Maintenance

After remission has been achieved, the goal is to maintain the symptom-free status, which can be accomplished with various medications, with the exception of glucocorticoids, which have no place in maintenance therapy, given the marked side effects associated with their long-term use. Both oral and rectal 5-aminosalicylate have greater efficacy than placebo for maintenance of remission in patients with distal disease [163]. Thiopurines (e.g., azathioprine at a dose of 2.5 mg per kilogram or 6-mercaptopurine at a dose of 1.5 mg per kilogram) are recommended when 5-aminosalicylate is ineffective or not tolerated or when the patient is glucocorticoid-dependent, although it may take several months before their maximal effectiveness is reached. For patients who do not have a response to immunosuppressive therapy or cannot tolerate it, anti–TNF-α agents are gradually being adopted; higher rates of remission and improvement on endoscopy, as well as lower rates of colectomy, are reported when infliximab trough levels are detectable in the circulation [164]. Unlike Crohn's disease, ulcerative colitis may respond to probiotic therapy. For example, *Escherichia coli strain Nissle 1917* (200 mg per day) is not less effective than 5-aminosalicylate (1.5 g perday) for maintaining remission [165], and the probiotic VSL#3 (3600 billion colony-forming units per day for 8 weeks) in conjunction with 5-aminosalicylate can help induce remission in mild-to moderate ulcerative colitis [166]. There are multiple indications for surgery, including the failure of medical therapy, intractable fulminant colitis, toxic megacolon, perforation, uncontrollable bleeding, intolerable side effects of medications, strictures that are not amenable to endoscopic alleviation unresectable high-grade or multifocal dysplasia, dysplasia-associated lesions or masses, cancer, and growth retardation in children[167]. There are also multiple surgical options. Traditional proctocolectomy with ileostomy is curative and technically straight forward; however, possible complications include small-bowel obstruction, fistulas, persistent pain, sexual and bladder dysfunction, and infertility [168]. Total proctocolectomy with ileal pouch–anal anastomosis (IPAA) is currently the procedure of choice for most patients who require elective surgery, since it has the distinct advantage of preserving anal-sphincter function. This approach is associated with an acceptable morbidity rate (19 to 27%), extremely low mortality (0.2 to 0.4%), and good postoperative quality of life [169]. Continent ileostomy is an alternative procedure for patients with

ulcerative colitis who are ineligible for or have declined IPAA or who have not been helped by it [169].

CHAPTER 3: PROBIOTICS, PREBIOTICS AND SYNBIOTICS

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 nonpathogenic, human origin; acid and bile resistance; survival of gastrointestinal transit; production of antimicrobial substances; and immune modulator activity [170,171]. The main probiotics on the market are lactobacilli, streptococci and bifidobacteria, which are normal constituents of the human gastrointestinal microflora. The first two belong to a large group of bacteria designated as lactic acid bacteria (LAB) [172].The genus Bifidobacterium is unrelated to LAB phylogenetically, and Bifidobacterium species use a unique metabolic pathway for sugar metabolism. However, Bifidobacterium species are often considered to be LAB and probiotics because of their documented health promoting effects [173]. 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 [174], 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.

Prebiotics

Prebiotics are indigestible carbohydrates that stimulate the growth and the activity of benefical bacteria, particularly lactobacilli and bifidobacteria [175]. Many years ago the prebiotic lactulose has been shown to improve symptoms in liver patients increasing the numbers of bifidobacteria [176] and today is commonly used in these patients [177]. 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 [178,179]. Any food that reaches the colon other than nondigestible carbohydrates, such as

peptides, proteins and 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) [180]. Because of the presence of the β-linkages, FOS are indigestible in the upper gastrointestinal tract. Consequently, they enter the cecum/large bowel intact, here they are largely fermented to short chain fatty acids (mainly acetate, propionate and butyrrate and other metabolites e.g. lactate) and cause proliferation of selected anaerobic bacteria, mostly bifidobacteria [180,181]. Thus, FOS including inulin, other oligosaccharides, lactulose, resistant starch and dietary fibres have been shown to promote a probiotic response [175]. Previously, it was also demonstrated that FOS modifying the gene expression of lipogenic enzyme, reduced *de novo* liver fatty acid synthesis [182], contributing to the decrease in TG accumulation in the liver. Studies provide novel insights on the possible link between prebiotics and metabolic diseases, such as obesity and IR [183,184]. Prebiotic supplementation is able to increase in plasma gut peptide concentrations (glucagon-like peptide 1 and peptide YY), which may contribute in part to the changes in satiety and post-prandial glycaemic response in healthy subjects [185]. 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 [186]. Indeed, the modification of intestinal microflora (increase in Bifidobacteria and subsequent reduction in Enterobacteriaceae), contributes to a reduction in faecal pH, which results in a minor rate of ammonia absorption and in a lower amount of total ammonia into the blood stream. 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.

Synbiotic

The term ‘synbiotic’ is used ‘when a product contains both probiotics and prebiotics’[187]. For example, the synbiotic combination of a specific oligofructose-enriched inulin (SYN1) 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* [188]. Recent *in vitro* studies have confirmed that synbiotic were more effective than prebiotics or probiotics in modulating the gut microflora [189].

3.1 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 strains. Understand the various mechanisms of probiotic action is crucial for the establishment of definitive selection criteria for certain strains or combination of strains for specific clinical conditions. Although the molecular mechanisms of probiotic are not completely elucidated, many effects could result beneficial in NAFLD, including the modulation of the intestinal microbiota, antibacterial substance production, epithelial barrier function, intestinal inflammation, or the immune system (**Figure 3.1**).

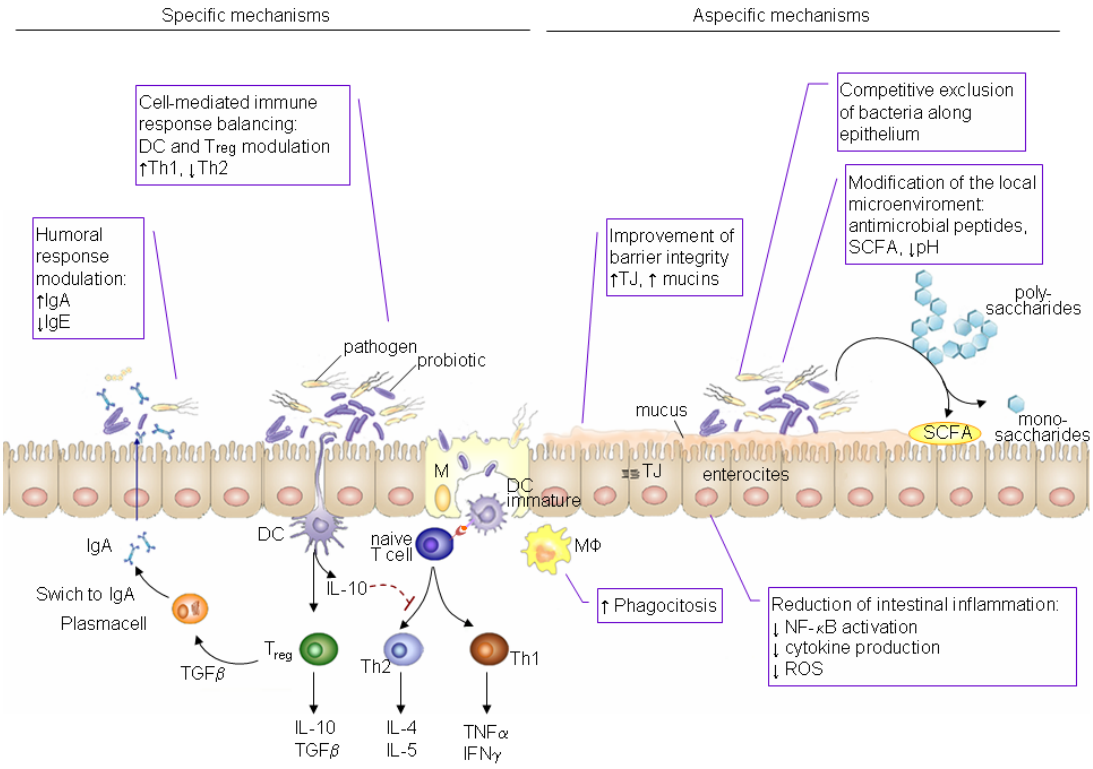


Fig. 3.1. Mechanisms of action of probiotics. Specific mechanisms: involvement of probiotics in cell-mediated and humoral immune responses. Aspecific mechanisms: enhancement of epithelial barrier function, competitive exclusion of bacteria along epithelium, modification of local microenvironment and 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. *Image from: Iacono A, Raso GM, Canani RB, Calignano A, Meli R. Probiotics as an emerging therapeutic strategy to treat NAFLD: focus on molecular and biochemical mechanisms. J Nutr Biochem. 2011 Aug;22(8):699-711. doi: 10.1016/j.jnutbio.2010.10.002. Epub 2011 Feb 2. Review.*

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. Non-specific antimicrobial substances include SCFAs [190], hydrogen peroxide [191], bacteriocins, bacteriocin-like inhibitory substances (BLIS), and bacteriophages [192]. 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, to dissociate in the more alkaline cell environment and to acidify the cytoplasm [193]. In microbial fermentor systems, pH modification could lead to a shift in the composition of the microbiota community [194], limiting the populations of certain gut pathogens [195]. Bacteriophages are highly specific and can be active against a single strain of bacteria. The 2-component lantibiotics, a class of bacteriocins produced by Gram-positive bacteria, such as *Lactococcus lactis,* are small antimicrobial peptides [196]. 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 [197]. 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 [198]. Bifidobacteria and Lactobacilli can adhere to intestinal epithelial cells through surface-expressed proteins [199]. In particular, *Lactobacillus casei* binds to extracellular matrix components, such as collagen, fibronectin or fibrinogen [200]. Moreover, a part 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 [201]. Several strains of lactobacilli and bifidobacteria are capable to compete with and displace pathogenic bacteria, including *Bacteroides vulgatus, Clostridium histolyticum, C. difficile, Enterobacter aerogenes, Listeria monocytogenes, Staphylococcus aureus, Salmonella enterica, Yersinia enterocolitica* [202], enterotoxigenic *E. coli* [203,204], and enteropathogenic *E. coli* [205], even if

the pathogens have attached to intestinal epithelial cells prior to probiotic treatment [202]. 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 [206,207], which are the same receptors mediating pathogenic bacteria binding to intestinal epithelial cells. For example, Lactobacilli and Bifibobacteria establish mannose and Galβ 1-3GalNAc-specific adhesions to attach to intestinal epithelial cells and mucus [206], 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, inhibiting pathogenic bacteria growth and/or competing with and displacing pathogenic bacteria can prevent small intestinal bacteria overgrowth.

Modification of intestinal epithelial permeability and function

Probiotics are able to improve the non-specific intestinal barrier defence mechanism, modulating tight junctional proteins and stimulating mucin production. These effects limits small intestinal bacterial overgrowth, and bacterial translocation, both events observed in humans and in animal models and responsible of the reduced endotoxemia [208]. The mucus layer, covering the gastrointestinal mucosa, is considered as the first line of defence against mechanical, chemical, or microbiological aggressions arising from the luminal contents. Indeed, the break of the mucus barrier in inflamed colon has been shown to allow bacterial adherence to epithelial tissue [209], and the removal of the mucus layer favours the penetration of high molecular weight probes in mucosa [210]. It has been demonstrated that Lactobacilli upregulate the MUC2 and MUC3 mucins and inhibit attachment of enterohemorrhagic *E.coli in vitro* [211] and that a probiotic mixture of lactobacilli and bifidobacteria increased the secretion of mucin, stimulating MUC2 gene expression in rat colon *in vivo* [212].

Probiotics stimulate the production of SCFAs [213], which, in turn, are able to modulate intestinal permeability as demonstrated several conditions, including antibiotic associated colitis, inflammatory bowel disease, colon cancer and hepatic encephalopathy. Probiotic administration could potentially reduce bacterial metabolites, that may be toxic to the intestinal epithelium for instance hydrogen sulphide and extracellular superoxide [214]. *Lactobacillus GG, Bifidobacterium infantis, Bifidobacterium lactis* and *E.coli Nissle 1917* increase tight junction integrity, preventing their disruption. The biochemical pathways mediating the probiotic effect on tight junction function include protein kinase C and MAP kinase pathways, and involve both redistribution and altered

expression of the tight junction proteins occludin, ZO-1 (zonula occludens-1) and ZO-2 and claudins 1, 2, 3 and 4 [215,216].

Modification of endotoxemia

The clear role for endotoxin levels in alcoholic liver injury, the involvement of endotoxemia in NAFLD has also been addressed. In fact, 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 MCD (methionine choline-deficient) diet, suggesting that TLR4 signalling is, indeed, important for the pathogenesis of NASH [217]. Moreover, depletion of Kupffer cells diminished diet-induced increases in TLR4 and TNF-α, indicating a crucial role for these cells in mediating TLR4 signalling and transcription of cytokines.

Suppression of inflammation

Intestinal inflammation leads to an increase of mucosal permeability and bacterial translocation. It is noteworthy that several cytokines, such as TNF-α, IFN-γ, IL-4 and IL-13 have been shown to increase permeability in vitro using intestinal epithelial monolayers [218], altering tight junction morphology and distribution [219], and thereby creating a self-perpetuating vicious cycle that amplify bacteria translocation and possibly extra-intestinal 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 can antagonise it within enterocytes by several mechanisms. In particular, the nuclear export of the p65 subunit of NF-κB can occur in a PPAR-γ-dependent manner [220]. Soluble components from a mixture of commercially available probiotics, VSL#3, and *Lactobacillus reuteri* inhibit epithelial proteasome function, preventing the degradation of IκB [219,221]. This was accompanied by an increased expression of nerve growth factor, which has anti-inflammatory properties. This finding implicates a role of the enteric nervous system in host-microbial interactions. A variety of probiotic bacteria including the mixture VSL#3, *L. reuteri, L. salivarius UCC118, and B. infantis 35624* have been shown to suppress IL-8 secretion from intestinal epithelial cells in response to several pathogenic bacteria [219,222]. This cytokine [223] transcriptionally regulated by NF-κB is a potent neutrophil-recruiting and activating chemokine. The anti-inflammatory effects of a number of probiotic bacteria including

Bifidobacterium infantis 35624 and *L. salivarius UCC118* have been shown also to be mediated, only in part, throught NF-κB [222]. Besides NF-κB pathway, other intracellular signal transduction pathways have also been associated with the protective effects mediated by probiotics. These include MAP Kinase, AP-1, and PPAR-γ pathways [224,225]. A part 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 [226,227]. The role of TNF-α in NAFLD has been well documented and was strengthened by the improvement in liver function with anti-TNF therapy [45]. 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 [45]. Similar data were obtained from our group in a model of NAFLD induced by a high fat diet; 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 COX-2 (Cyclooxygenase-2) and iNOS (inducible nitric oxide synthase) expression. This effects was also evidenced by VSL#3 capability to reduce hepatic TNF-α level, the key pathogenetic factor responsible of the onset of NASH, and restoring PPAR-α expression [70]. Another study measured hepatic natural killer T (NKT)-cell depletion in high-fat fed animals. This diet induced the depletion of NKT from the liver, leading overproduction of TNF-α and causing inflammation, insulin resistance and steatosis. VSL#3 significantly improve all these parameters restoring insulin signalling [228]. Considering the anti-inflammatory properties of more than 550 different lactic acid bacteria strains, a new synbiotic composition was obtained, consisting in *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, Synbiotic 2000, was successfully investigated in surgical operations such as liver transplantation reducing the problem of postoperative infections [229].

Immune system modulation by probiotics

Commensal bacteria can modulate the immune system both at local and systemic level. Signals mediated by these bacteria are essential for optimal mucosal and immune development, and to maintain or restore gut integrity [230,231]. 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 (lipopolysaccharide, 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 [232], which share the same host-microbial signalling 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 [233]. 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 TGF-β (transforming growth factor beta) [234]. 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 [235,236]. DCs from different lymphoid compartments exhibit divergent cytokine responses to probiotic and pathogenic bacteria [237]. Some strains of probiotic bacteria, such as *L. casei* or *L. reuteri*, but not *L. plantarum*, can promote DCs to induce tolerance driving the development of regulatory T cells [238]. 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 [239]. Probiotics can interact either directly with DCs or indirectly, via the action of M cells. Very recently, it has been evaluated the ability of three lactobacilli strains (*Plantarum, LGG and paracasei B21060*) to activate DCs. *L. paracasei B21060* was identified as the more immunomodulatory among the three strains, and was able to inhibit the inflammatory potential of pathogenic *Salmonella* and protect against experimental colitis [240]. Probiotics, in addition to facilitate cell mediated immunity promote humoral response. The administration of probiotic bacteria leads to an increase in the levels of pathogen-specific IgA [241], and IgA responses are enhanced in formula-fed infants supplemented with probiotics compared with infants receiving placebo [242]. Of note, the induction of IgA in the gut is heavily dependent on TGF-β, which is also closely involved in the maturation of regulatory T cells [243]. In agreement with these studies, a recent randomised, double-blind, placebo-controlled trial demonstrated that the administration of two probiotic bacteria, *L. gasseri CECT5714* and *L. coryniformis*, increased the proportion and activity of phagocytic and NKT cells, as well as levels of IgA in healthy adults [244]. Particularly

desirable strains are those that improve immune function by increasing the number of IgA-producing plasma cells, improve phagocytosis, and the proportion of Th1 cells and NKT cells [245]. Some strains are more likely to have strong clinical effects; among them are strains like *L. paracasei subsp paracasei, L. plantarum, and Pediococcus pentosaceus*. In particular, *L. 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 [246], suppress splenocytes proliferation [247] and decrease antigen-specific IgE and IgG1 [248]. *L. paracasei* was also shown to be the strongest inducer of Th1 and repressor of Th2 cytokines [249]. Moreover, it has been shown that co-culturing lactic acid bacteria with human or rodent leukocytes has been shown to augment the production of type II interferon (IFN-γ by mitogen-stimulated mononuclear cells), or to induce type I interferon (IFN-α production by isolated macrophages) [250]. Both interferons promote Th1-type immune responses and reduce IgE production [251]. IL-12 has been shown to be an important pro-interferon cytokine involved in the production of lactic acid bacteria-stimulated IFN-γ [250]. 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 IFN-γ 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 [252]. The varying immunological effects of bacteria highlight differences that arise when different type, fluid, or tissues are used.

Probiotics efficacy in NAFLD: from animal models to clinical evidences

The major difficulties in our knowledge on probiotics efficacy in NAFLD derived from the different experimental models used and bacterial strains tested. Clinical research into mechanisms of NAFLD development and progression are limited by ethical considerations, particularly with respect to obtaining liver and other tissues, and by inadequate ability to delineate cause and effect from complex pathology with several involved mechanisms. It is therefore attractive experimentally 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 [253]. The central features of the "modern life style" that predisposes to overweight, obesity, insulin resistance and fatty liver disease is the constant caloric overconsumption, also known as "over nutrition". Over nutrition has been achieved in animal models in a number of different ways, including forced

feeding, administration of high-fat diets, the use of genetically hyperphagic animals, or a combination of these approaches. The effects of administering a high fat diet 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 could range between 40% and 70%. The well known study by Lieber and colleagues described the effects of feeding a liquid high-fat diet (HFD) to Sprague–Dawley rats [254]. High-fat-fed rats showed quickly extensive mitochondrial abnormalities and dysfunction producing reactive oxygen species with an array of responses that results 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 [255] creating in several weeks a model of IR and NAFLD/NASH in non-genetically modified animals [256]. This model is characterized by visceral obesity, increased glucose and insulin levels, decreased PPAR-α expression, and alterations in insulin signalling 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 "over nutrition" 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 3.1**. As depicted, the most characterized probiotic is VSL#3 mixture, active in several murine models of high fat diet-induced NAFLD/NASH. Li et al. [45] using *ob/ob* mice fed with a HFD provided the first evidence that manipulation of the intestinal flora in 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 of the JNK and NF-kB activity, of fatty acid β-oxidation and of mitochondrial uncoupling protein (UCP)-2 expression, all markers and factors characterizing IR. Subsequently, Ma et al. [228] showed that oral VSL#3 treatment significantly improved the HFD-induced IR and steatosis recovering hepatic NKT cell depletion. Conversely, recent data have demonstrated that in another model of NAFLD/NASH, VSL#3 attenuated fibrosis, reducing TGF-β and collagen, α-SMA (alpha smooth muscle actin), MMPs expression, but had no effect on liver steatosis parameters and inflammation in MCD-fed mice [112]. These data are limited by the type of diet used in these animal models. The major disadvantage of the MCD model is that it is associated with significant weight loss, low serum leptin level and peripheral insulin sensitivity.

Table 3.1. Effect of several probiotics in experimental model of NAFLD.

Probiotic	Experimental model	Duration of therapy	Results	Reference
VSL#3 1.5×10^9 CFU/mouse/day	Mice: *ob/ob* mice fed high-fat diet (HFD)	4 wks	Improved NAFLD histology and reduction of hepatic total fatty acid content, and serum ALT levels; amelioration of the hepatic insulin resistance	[45]
Bacillus polyfermenticus SCD 3.1×10^6 CFU/day	Rats: high-fat and high-cholesterol diet	6 wks	Reduction in plasma LDL, cholesterol, and hepatic total cholesterol, and triglycerides,	[257]
Lactobacillus rhamnosus PL60 1.0×10^7 -1.0×10^9 CFU/mouse/day	Mice: high-fat diet (HFD)	8 wks	Resolution of hepatic steatosis (at higher dose)	[258]
Lactobacillus acidophilus and L. casei	Rats: high fructose diet	8 wks	Reduced liver oxidative stress, improved insulin resistance	[259]
VSL#3 1.5×10^9 CFU/mouse/day	Mice: high-fat diet (HFD)	4 wks	Improved HFD-induced hepatic NKT cell depletion, insulin resistance and hepatic steatosis and inflammation	[228]
Lactobacillus plantarum MA2 1×10^{11} CFU/rat/day	Rats: cholesterol-enriched diet	5 wks	Reduction in liver and serum cholesterol and triglycerides	[260]
VSL#3 1.3×10^{10} CFU/kg	Rats: high-fat diet (HFD)	4 wks	Amelioration of the hepatic inflammatory, steatotic and peroxidative factors and reduction in serum aminotransferase levels	[70]
VSL#3 in drinking water	Mice: methionine-choline-deficient (MCD) diet	9 wks	No effect on MCD-induced liver steatosis and inflammation, but amelioration of liver fibrosis	[112]
Lactobacillus paracasei B21060 2.5×10^8 bacteria/ kg/ die	Rats: high fat diet	6 wks	Ameliorated steatosis, improved insulin resistance, decreased hepatic inflammatory cytokines.	[113]

From: Iacono A, Raso GM, Canani RB, Calignano A, Meli R. Probiotics as an emerging therapeutic strategy to treat NAFLD: focus on molecular and biochemical mechanisms. J Nutr Biochem. 2011 Aug;22(8):699-711. doi: 10.1016/j.jnutbio.2010.10.002. Epub 2011 Feb 2. Review.

The severe atrophy of adipose tissue in MCD-fed mice suggests that in this model NASH reflects the associated lipodystrophy rather than metabolic syndrome [261]. Among probiotics, several strains of *lactobacillus* have shown to have a protective effect on NAFLD [260]. In particular, an eight-week oral treatment with *Lactobacillus rhamnosus PL60* showed an anti-obesity effect and liver steatosis in DIO (diet inducing obesity) mice. Histopathological analysis of liver steatosis evidenced a lowered grading score in DIO mice receiving *L. rhamnosus* [258]. Moreover, a beneficial effect on liver alteration has been shown in *L. acidophilus* and *L. casei* treated mice fed with a high fructose-diet. This diet, indeed, provides 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, inducing, other than IR, steatosis. In this study, these two probiotics delayed the onset of glucose intolerance, reduced insulinemia and liver glycogen and ameliorated the steatosis, reducing MDA (malonyl dialdehyde) and increasing GSH (Glutathione) content [259].

Using a cholesterol enriched diet, Wang et al.[260] demonstrated that the administration of *L. 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 were previously observed when Bacillus polyfermenticus was administered in rat fed with high fat and high cholesterol diet [257]. 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 about their efficacy in NAFLD in humans. The first study [262] tested a mixture of probiotics (*Lactobacillus acidophilus, Bifidus, Rhamnosus, Plantarum, Salivarius, Bulgaricus, Lactis, Casei, Breve*) associated with prebiotics FOS and vitamins (B6, B2, B12, D3, C and folic acid) in ten patients with biopsy-proven NASH. After two 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 induced by alcohol or HCV [263]. Independently by pathogenesisis of liver disease, VSL#3 administered for three months, significantly improved plasma levels of MDA and 4-hydroxynonenal (4-HNE), both markers of lipid peroxidation; whereas cytokines (TNF-α, IL-6 and IL-10) were reduced only in alcoholi dependent patients. S-nitrosothiols (S-NO) plasma levels were improved at the end treatment in all groups. These promising preliminary results strongly suggest a great potential for probiotics use in 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 [264]. The large amount of experimental data on probiotics effects that is now available could drive in the next future the design of clinical trials.

3.2 Biological and molecular basis of probiotic action in IBD

Role of the commensal flora in IBD

Although many studies have investigated the possibility of a single infectious agent causing Crohn's disease and ulcerative colitis, also called chronic IBD, none has yet been discovered. The intestinal bacteria are now believed to be involved in the initiation and perpetuation of IBD. The prevailing theory explaining the development of IBD is that the adaptive immune system is hyper-responsive to the commensal intestinal microflora in genetically susceptible individuals [265]. This hypothesis is supported by several observations: most inflammation occurs in areas with the highest density of intestinal bacteria, broad spectrum antibiotics improve chronic intestinal inflammation, and surgical diversion of the fecal stream can prevent recurrence of Crohn's disease. Most importantly, despite differences in the pathogenesis of chronic intestinal inflammation, a consistent feature of many animal models of IBD (such as IL-10 knockout mice and HLA-B27 transgenic rats) is the failure to develop chronic intestinal inflammation when these animals are raised in germ-free conditions [266,267]. Dysbiosis is also observed in IBD patients. Adherent and intramucosal bacteria, particularly Bacteroides spp, *Escherichia coli* and Enterobacterium spp are more abundant in patients with Crohn's disease than in controls [268,269]. Bacterial overgrowth and dysbiosis are also associated with the development of chronic pouchitis, the inflammation of the ileal reservoir created after ileo-anal anastamosis following colectomy in ulcerative colitis patients [270]. In addition, several selected commensal bacterial species can induce and perpetuate colitis in genetically susceptible rodent models of chronic intestinal inflammation [271]. The recognition of the compelling association between intestinal microflora and the development of IBD has led to an abundance of studies investigating the therapeutic potential of altering luminal bacteria using probiotics and/or prebiotics (**Figure 3.2**).

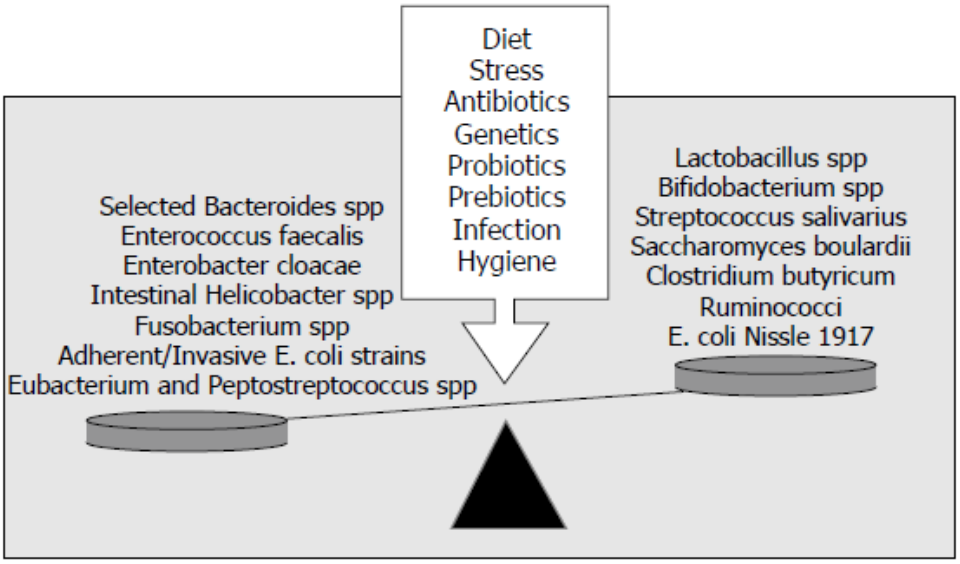


Fig. 3.2. Microbial balance and dysbiosis. The pathogenic immune responses present in IBD are triggered by the presence of luminal bacteria. The balance of beneficial vs aggressive intestinal microbes is responsible for either mucosal homeostasis or chronic inflammation. A number of environmental and genetic factors influence the balance of beneficial vs aggressive microbes. *From: Ewaschuk JB, Dieleman LA. Probiotics and prebiotics in chronic inflammatory bowel diseases. World J Gastroenterol. 2006 Oct 7;12(37):5941-50.*

Protective mechanisms of probiotics by ameliorating chronic intestinal inflammation

Probiotic bacteria have beneficial effects on the intestinal epithelium both directly and indirectly, including enhanced barrier function, modulation of the mucosal immune system, production of antimicrobials, and alteration of the intestinal microflora.

Alteration of the mucosal immune system

The presence of probiotics has been shown to result in several modifications in the mucosal immune response, including augmented antibody production [272], increased phagocyte [273] and natural killer cell activity [244,274], modulation of NF-κB pathway [171,221], and induction of T cell apoptosis [275]. Generally, probiotics increase the production of intestinal anti-inflammatory cytokines (such as IL-10 and TGF-β), while reducing the production of pro-inflammatory cytokines (e.g., TNF-α, IFN-γ, IL-8) [276,277]. Several probiotic bacteria, including *B. breve, Streptococcus thermophilus, B. bifidum* and *Ruminococcus gnavus* have been shown to secrete metabolites that reduce LPS-induced TNF-α secretion [278]. *L. reuteri* reduces TNF-α and *Salmonella typhimurium*

induces IL-8 secretion in vitro, by inhibiting nuclear translocation of NF-κB and preventing the degradation of IκB [219]. Administration of the probiotic cocktail VSL#3 (consisting of *L. acidophilus, L. bulgaricus, L. casei, L, plantarum, B. breve, B. infantis, B. longum, S. thermophilus*) to IL-10 deficient mice results in colitis reduction and a concomitant reduction in mucosal secretion of TNF-α IFN-γ [279]. *E. coli Nissle 1917* is able to down-regulate the expansion of newly recruited T-cells into the mucosa and limit chronic intestinal inflammation [280]. In SAMP1/Yit mice, *Lactobacillus casei strain Shirota* inhibits IL-6 production in LPS-stimulated large intestinal lamina propria mononuclear cells and down-regulates nuclear translocation of NF-κB [281]. Patients with a recent ileo-anal pouch anastomosis who responded to probiotic therapy have reduced mRNA levels of IL-1β, IL-8 and IFN-γ, and fewer polymorphonuclear cells, compared with patients receiving placebo[282]. Probiotic treatment has also been shown to reduce IFN-γ and IL-1α expression and decrease inducible-nitric oxide synthase and gelatinase activities in pouch biopsy samples from patients with pouchitis [283]. In mucosal explants of ileal specimens from patients with Crohn's disease, probiotics reduced TNF-α release and the number of CD4 cells [284]. In addition to live probiotics, components of probiotic bacteria can also exert effects on the mucosal immune system. For example, genomic DNA isolated from VSL#3 inhibits TNF-α-induced IL-8 secretion, mitogen-activated protein kinase activation and NF-κB activation [171] in HT-29 cells.

Improved barrier function

Various probiotic bacteria can enhance intestinal epithelial barrier function. For example, oral administration of VSL#3 results in normalization of impaired colonic barrier function and restoration of intestinal epithelial integrity in IL-10 deficient mice and enhancement of epithelial resistance in T-84 cells [279]. Barrier function was enhanced not only by live bacteria, but also by a proteinaceous secreted product of VSL#3 [279]. Several strains of lactobacilli are also capable of upregulating intestinal MUC3 mRNA expression, thereby improving barrier function by increasing the mucus layer [285]. *Lactobacillus GG* improves barrier function by inhibiting apoptosis of intestinal epithelial cells [286]. *S. thermophilus and L. acidophilus* have been shown to enhance phosphorylation of actinin and occludin in the tight junction, thereby preventing the invasion of enteroinvasive *E. coli* into human intestinal epithelial cells [287].

Alteration of the intestinal flora

Probiotics suppress the growth and invasion of pathogens in several ways. They competitively exclude pathogenic bacteria by occupying the limited physical space in the mucus layer and on epithelial cells. They also engage pattern-recognition receptors and consume substrate otherwise available to other (pathogenic) microbes. In addition, probiotics render their microenvironment inauspicious for pathogens by secreting antimicrobial substances such as hydrogen peroxide, organic acids, and bacteriocins. For example, both in vitro and in vivo experiments demonstrate that *B. Infantis* suppresses the growth of *Bacteroides vulgates* [288]. Patients with pouchitis treated with VSL#3 have been demonstrated to have increased bacterial diversity in the pouch, and decreased fungal diversity [289]. Probiotics may also alter the intestinal microflora by changing the fatty acid profile in the colon. VSL#3 probiotic strains are also capable of converting linoleic acid to conjugated linoleic acid, a fatty acid with anti-inflammatory and anti-carcinogenic properties [225].

Therapeutic efficacy of probiotics in IBD treatment

Results from various animal studies and clinical trials using probiotics to treat intestinal inflammation have generated considerable excitement. Data are now emerging which suggest that probiotics are capable of preventing relapse of chronic intestinal inflammation. Some probiotics can even treat mild to moderately active IBD [170,290]. Although there is a paucity of human studies using prebiotics, the few emerging studies showed that there is potential for this treatment modality. A multi-center open-label trial reported that oral administration of GBF (germinated barley foodstuff) to patients with mild to moderately active UC for 24 wk resulted in a significant decrease in clinical activity index, compared to controls [291]. An open-label study of 22 UC patients in remission showed that a daily oral intake of 20 g GBF resulted in a significantly improved clinical activity index and endoscopic score at 3, 6 and 12 mo, and a reduced relapse rate, compared with controls [292]. A recent randomized, double-blinded controlled trial by Furrie et al [293] examined the use of synbiotics in 18 patients with active UC, using a combination therapy of *B. longum*, inulin and oligofructose, and found that sigmoidoscopy inflammation scores are reduced in the synbiotic-treated population when compared to placebo. Intestinal levels of TNF-α and IL-1β are also reduced. Additionally, rectal biopsies have demonstrated reduced inflammation and greater epithelial regeneration in the synbiotic-treatment group.

Adverse effect of probiotics

Probiotics are generally regarded as safe. Side effects are rarely reported and generally amount to little more than flatulence or change in bowel habit. A review outlining the safety of current probiotic compounds has been published [294]. The use of probiotics in immunocompromised or in critical ill patients should be carefully evaluated to limit the risk of endocariditis or sepsis. However, cases of infection caused by lactobacilli and bifidobacteria are extremely rare and are estimated to occur in approximately 0.05% to 0.4% of all cases of infective endocarditis and bacteraemia [294]. One important clinical characteristic of lactobacilli is their resistance to antibiotic vancomycin, empirically used against Gram negative bacteraemia. Lactobacilli are considered as emerging pathogens in high-risk patients with neutropenia induced by chemotherapy [295], in neonates submitted to surgery on a count of cardiovascular disorders in paediatric patients submitted to gastrojejunostomy [296]. No increase in bacteraemia caused by Lactobacillus species was seen in Finland over the period of 1990–2000, despite an increased consumption of *L. rhamnosus GG*. A study of long-term consumption of *Bifidobacterium lactis* and *S. thermophilus*-supplemented formula in children aged less than 2 years showed the product was well tolerated [297]. Complications of treatment with probiotics have been observed in patients who are immunocompromised or in the intensive care setting. S. cerevisae fungaemia [298] and Lactobacillus bacteraemia [296,299] have been reported in patients with severe underlying illnesses. Nevertheless, case reports have identified fungemia in two immunosuppressed patients [298] and exacerbation of diarrhoea in two patients with ulcerative colitis who consumed *S. boulardii* [300].

CHAPTER 4: SHORT CHAIN FATTY ACIDS (SCFAs)

Production

SCFAs are organic fatty acids with 1 to 6 carbon atoms and are the principal anions which arise from bacterial fermentation of polysaccharide, oligosaccharide, protein, peptide, and glycoprotein precursors in the colon [301,302]. Fermentation involves a variety of reactions and metabolic processes in the anaerobic microbial breakdown of organic matter, yielding metabolizable energy for microbial growth and maintenance and other metabolic end products for use by the host. The chief end products are SCFAs together with gases (CO_2, CH_4, and H_2) and heat [303]. Various population survey data show that fecal SCFA production is in the order of acetate> propionate> butyrate in a molar ratio of approximately 60:20:20, respectively [304]. The ratio seems to remain fairly constant [22], although alterations in production and absorption may occur with dietary changes.

Carbohydrates are fermented by saccarolytic bacteria primarily in the proximal colon producing linear SCFAs, H_2, and CO_2 [305], and both the presence of carbohydrates in the colon and their fermentation can alter the colonic physiology. Fermentation of proteins and amino acids by proteolytic bacteria yield branched SCFAs, H_2, CO_2, CH_4, phenols, and amines. The primary effects of SCFAs are on colonic function as a result of their uptake and metabolism by colonocytes, although SCFAs are also metabolic substrates for other tissues of the host.

The production of SCFAs are determined by a number of factors, including the numbers and types of microflora present in the colon, substrate source [306], and intestinal transit time [306,307]. A large microflora population is present in the human colon at 10^{10} to 10^{11} cfu/g wet weight [308], and more than 50 genera and over 400 species of bacteria have been identified in human feces [181,309]. Bacterial numbers, fermentation, and proliferation are highest in the proximal colon where substrate availability is greatest [310]. The principal site of colonic fermentation, therefore, is the cecum and proximal colon, whereas the distal colon is carbohydrate and water depleted. The total amount of SCFA in the proximal colon is estimated to range from 70 to 140 mM [303,306] and fall to 20 to 70 mM in the distal colon [303]. Therefore, the pH is lowest in the proximal colon and increases distally. Samples at various sites taken from patients with colonostomies indicate a decline in SCFA levels along the colon [303]. Specific species such as Bifidobacterium and Lactobacillus have been associated with improved health, resulting in the emergence of the sciences of probiotics, or delivery of specific bacteria to the colon and prebiotics, or the administration of

dietary components that promote the growth of specific bacteria with defined metabolic functions. The production of SCFAs are also determined by the substrate source where dietary intake is the most important variable. Nondigestible food components are a source of substrate for fermentation by anerobic colonic microflora, because they are resistant to hydrolysis and digestion in the stomach and small intestine and eventually enter the colon for fermentation. Carbohydrates quantitatively play the most important role in the formation of SCFA [303,305]. On transit through the colon, substrates available for fermentation are depleted, as reflected in the decline in SCFA production [310] and in a range of other physiologic effects. Furthermore, substrate availability during gut transit may also change the bacterial populations and numbers [309]. Neither total SCFA nor the individual acids in the distal colon are predictive of those found proximally [311,312]. SCFA availability in the distal colon is dynamic as water absorption and loss of digestive material alter availability of SCFAs independent of rates of production. A curvilinear relationship exists between intestinal transit time and fecal total and individual SCFAs (especially butyrate) so that at whole gut transit times >50 hours, butyrate cannot be detected, likely because of colonic uptake [303]. Total SCFA and regional differences in SCFA concentration are implicated in diseases of the colon, especially in cancer and gastrointestinal disorders, where disease often occurs distally. Therefore, increased SCFA production and a greater delivery of SCFA distally, especially butyrate, may have a role in preventing these diseases.

Carbohydrate and dietary fiber fermentation

SCFAs are produced from the fermentation of carbohydrates with the major source coming from resistant starches [313,314]. However, dietary fiber, unabsorbed sugars, raffinose, starchyose, polydextrose, and modified cellulose also represent significant sources of fermentable substrates in the colon [313]. It is estimated that 5% to 20% of dietary starch is not absorbed by the human small intestine [315,316]. Insoluble fibers (eg, lignins, cellulose, and some hemicelluloses), which are resistant to fermentation by colonic microflora play an important role in fecal bulking and may carry with them fermentable carbohydrate substrate, including starches and sugars [317]. Soluble fibers (eg, pectins, gums, mucilages, and some hemicelluloses) are more completely fermented by colonic microflora and may have little effect in increasing fecal bulk. When transit and laxation are unchanged, a greater intake of fermentable carbohydrates will result in higher SCFA production because of increased substrate availability [318,319].Concentrations and excretion of SCFA have been shown to be greater with feeding of some nonstarch polysaccharides, such as partially

hydrolyzed guar gum but not others such as oat bran [319]. Therefore, there are several factors that may affect substrate fermentability that complicate their use in human studies, coupled along with a limited ability to measure SCFA directly at specific sites in the colon. Polyfructans are fermented by colonic bacteria [320,321] specifically the Bifidobacterium species [320], which have been shown to be associated with serum low density lipoprotein- cholesterol (LDL-C) reduction [322]. Furthermore, fermentation end products of polyfructans, specifically the SCFA propionate, have been shown to decrease the acetate:propionate ratio when compared with lactulose [323] and to reduce serum cholesterol levels. Hence, there is a potential use for polyfructans (eg, inulin and oligofructose) in combination with viscous fibers (eg, oat bran) to lower serum cholesterol. Such combination effects on colonic microflora may be used to achieve a range of therapeutic and preventive effects. Various sources of resistant starches [318,319] and acarbose, the α–glycoside hydrolase inhibitor [324,325] also raise fecal SCFAs. The increases in these studies have either been reported as higher concentrations, excretion or both, which may reflect changes in production, absorption, and intestinal transit time.

Changes in SCFA may not be observed where significant quantities of lower doses of nondigestible oligosaccharides are given, which would be rapidly fermented and immediately absorbed in the more proximal colon [303]. Production of individual SCFAs have also been measured in various studies. Greater fecal excretion of butyrate and propionate have been observed with consumption of wheat bran compared with vegetable fiber [326]. However, feeding of partially hydrolyzed guar gum resulted in greater fecal excretion of all 3 major SCFAs, but did not change the concentration of propionate and butyrate, or decrease their relative contribution. Studies using resistant starch have been consistent in showing raised fecal butyrate [317,319]. Starch fermentation primarily yields acetate and butyrate, whereas fermentation of pectin and xylan yields acetate alone as the main product. Recent human studies found that acute ingestion of a nondigestible monosaccharide, L-rhamnose (25 g), increased serum propionate without increasing acetate [327], and this effect did not diminish after 28 days [328].

Absorption

Absorption of SCFAs in the cecum and the colon is a very efficient process with only 5% to 10% being excreted in the feces [329]. Two proposed mechanisms of absorption are (1) diffusion of protonated SCFAs and (2) anion exchange [306]. SCFAs are rapidly absorbed in the colon, which is associated with enhanced sodium absorption and bicarbonate excretion [306]. Intubation studies

have shown that SCFAs are taken up from the perfused human large bowel in a concentration-dependent manner [330]. At least 60% of that uptake is by simple diffusion of protonated SCFAs involving hydration of luminal CO_2, whereas the remainder occurs by cellular uptake of ionized SCFAs involving transport of Na^+ and K^+ [331]. SCFA uptake is associated with transport of water that seems to be greater in the distal than in the proximal colon [332]. Human peripheral venous blood concentrations of SCFAs are normally low, and only acetate is present in significant amounts [303]. However, Wolever et al., [333] measured serum propionate and butyrate, and reported values of 4.5 to 6.6 mmol/L and 2.2 to 3.9 mmol/L, respectively.

The major SCFAs: acetate, propionate, and butyrate are absorbed at comparable rates in different regions of the colon [330]. Once absorbed, SCFAs are metabolized at 3 major sites in the body: (1) cells of the ceco-colonic epithelium that use butyrate as a major substrate for maintenance-energy producing pathways; (2) liver cells that metabolize residual butyrate with propionate used for gluconeogenesis and 50% to 70% of acetate also taken up by the liver; (3) muscle cells that generate energy from the oxidation of residual acetate. Their oxidation supplies some 60% to 70% of the energy needs of isolated colonocytes [334], reduces glucose oxidation [335], and spares pyruvate [336] and glutamine [337]. In the presence of competing substrates such as glucose and glutamine, butyrate is the preferred intestinal fuel [338] suggesting that a hierarchy of oxidation exists with butyrate apparently being oxidized more in the proximal than in the distal colon [303].

Function of SCFAs

The role of SCFAs has expanded to include their role as nutrients for the colonic epithelium, as modulators of colonic and intracellular pH, cell volume, and other functions associated with ion transport, and as regulators of proliferation, differentiation, and gene expression [306]. Increases in SCFAs result in decreased pH, which indirectly influences the composition of the colonic microflora (eg, reduces potentially pathogenic clostridia when pH is more acidic), decreases solubility of bile acids, increases absorption of minerals (indirectly), and reduces the ammonia absorption by the protonic dissociation of ammonia and other amines (ie, the formation of the less diffusible NH_4^+ compared with the diffusible NH_3) [339]. In **figure 4.1** the structural form of main SCFAs are shown.

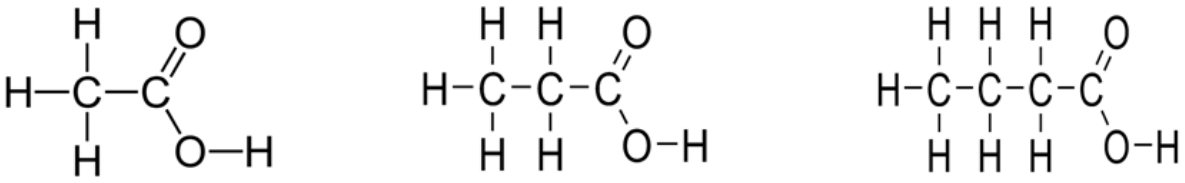


Figure 4.1 : the chemical structure of main SCFAs.

Acetate

Acetate, the principal SCFA in the colon, is readily absorbed and transported to the liver, and therefore is less metabolized in the colon [306]. The presence of acetyl-CoA synthetase in the cytosol of adipose and mammary glands allow the use of acetate for lipogenesis once it enters the systemic circulation. In human studies, acetate is often used to monitor colonic events because it is the main SCFA in the blood. Acetate is the primary substrate for cholesterol synthesis. Subjects given rectal infusions of acetate and propionate showed a dose-dependent increase in serum total cholesterol and triglyceride levels, providing indirect evidence that SCFA is utilized for lipid synthesis [340]. However, the methodology used in this study may have resulted in nonphysiologic levels of acetyl CoA from the rapid uptake of acetate. This may have diverted SCFA to lipid synthesis rather than oxidation [341]. It is possible that substrate-dependent SCFA produced by fermentation inhibits cholesterol synthesis [342]. However, uniform agreement has not been reached on the effect of increased colonic fermentation on lipid metabolism, because the possibility exists that different substrates may produce different effects [340]. However, results from human studies have been inconsistent. One-week intakes of 2.7 g sodium propionate taken as a capsule [343] did not affect serum lipids. Only one study showed that 5.4 g of propionate given daily for 2 weeks lowered LDL-C and total cholesterol in subjects with total cholesterol>5.5 mmol/L [324]. Studies using rectal infusions indicated that 180 mmol of propionate did not affect serum lipids or triglycerides in healthy young men and women. However, when 60 mmol of propionate was infused with 180 mmol of acetate, free fatty acids decreased by an additional 10% and negated the increase in total and LDL-C seen when acetate was given alone [344]. Therefore, it still seems possible that one of the determinants of the actions of propionate on serum lipids is the ratio of propionate to acetate [345,346].

Propionate

Propionate is produced via 2 main pathways: (1) fixation of CO_2 to form succinate, which is subsequently decarboxylated (the ‘‘dicarboxylic acid pathway’’); (2) from lactate and acrylate (the ‘‘acrylate pathway’’) [22]. Propionate is a substrate for hepatic gluconeogenesis and has been reported to inhibit cholesterol synthesis in hepatic tissue [343]. However, propionate seems to have two competing and opposite effects on gluconeogenesis. It is both a substrate for gluconeogenesis and an inhibitor of gluconeogenesis. Propionate enters the Krebs cycle at the level of succinyl CoA. The inhibiting effect of propionate on gluconeogenesis may be related to its metabolic intermediaries, methymalonyl CoA and succinyl CoA, which are specific inhibitors of pyruvate carboxylase. Propionate enhances glycolysis, probably by depleting hepatic citrate, which is an important metabolic inhibitor of phosphofructokinase. Propionate may also influence hepatic glucose metabolism indirectly by lowering the plasma fatty acid concentration, which, in itself, is known to be closely related to the actual rate of gluconeogenesis [347].

The majority of our knowledge about the nutritional fate of propionate comes from studies in ruminants. Intestinal glucose uptake is minimal in ruminants because of the presence of microbiota in their rumen for the digestion and fermentation of carbohydrates. However, propionate metabolism in humans is less well understood. In humans, propionate may also have systemic effects, specifically a hypolipidemic action. Observations in animals suggest that propionate inhibits cholesterol synthesis by inhibiting both 3-hydroxy-3-methylglutaryl- CoA synthase and 3-hydroxy-3-methylglutaryl-CoA reductase [348]. As previously mentioned, polyfructans are bifidogenic and decrease the acetate: propionate ratio, both of which are associated with reductions in serum lipids. The use of polyfructans (eg, Neosugar, inulin) in individuals with Type 2 diabetes mellitus (8 g/d) [349] and hyperlipidemia (18 g/d) [350] resulted in cholesterol reductions. However, no hypolipidemic effect (20 g/d) was observed in healthy subjects [323]. This inconsistency in human intervention studies, in contrast to animal experiments, may be related to species differences. A number of mechanisms have been suggested to be responsible for the observed lipid lowering effect, with increased propionate production being one of the possible mechanisms of action. Increased production of propionate, through fermentation, may inhibit hepatic cholesterol synthesis [344,345]. This has been supported in studies with hyperlipidemic experimental animals [342] but not supported in other animal studies [351]. Currently, there are limited human experimental data that have quantified the synthesis of acetate and propionate with use of prebiotics. Propionate is better absorbed in the human colon than acetate [352], and studies in ruminant mucosa show that propionate is activated to its coenzyme A derivative (a step required for its oxidation) to a greater

extent than acetate. The liver extracts 90% of propionate, as opposed to 75% of acetate, during a single pass [334] and colon infusions of equal amounts of acetate and propionate suggest that the amount of colonic propionate reaching peripheral blood is only 25% of the amount of total colonic acetate.

Butyrate

Butyrate is the preferred fuel of the colonic epithelial cells which also plays a major role in regulation of cell proliferation and differentiation [303,353]. It is the most important SCFA in colonocyte metabolism, where 70% to 90% of butyrate is metabolized by the colonocyte [306]. Butyrate is used preferentially over propionate and acetate in a ratio of 90:30:50 [306], and is preferred over glucose or glutamine supplied by blood [354]. Butyrate oxidation has been shown to make up more than 70% of the oxygen consumed by human colonic tissue. Sodium butyrate exerts an antiproliferative activity on many cell types, and there are evidence from animal and cell line studies, that have demonstrated preventive effects of butyrate on colon cancer and adenoma development [355]. Acetate and propionate have also been shown to induce apoptosis in colorectal tumor cell lines, but to a much lesser extent than butyrate [356]. Butyrate also stimulates immunogenicity of cancer cells [357]. Currently, the mechanisms of action of butyrate in relation to colon cancer are not clearly defined. Butyrate induces p21WAFI/Cip1 protein and mRNA levels [358], which can block the cell cycle at G1, resulting in the inhibition of cell proliferation. This blockage of the cell cycle at G1 might allow DNA checkpoint-mediated repair of genomic instability or mutations [359]. Through inhibition of histone deacetylase, butyrate has been shown to induce apoptosis through hyperacetylation of histones (H3 and H4) [360], resulting in the DNA being in a more open form [361]. The open form of the DNA would be ideal if DNA damage had occurred and repair enzymes were necessary to approach the damaged DNA. However, the open form of the DNA may be more susceptible to mutation in the presence of a carcinogen [362]. The ability of butyrate to inhibit histone deacetylase may also have a role in reversing epigenetic events [363]. *In vitro*, butyrate can also induce differentiation of neoplastic colonocytes producing a phenotype typically associated with normal mature cells [363]. The drop in colonic pH caused by accumulation of SCFAs decrease the solubility of free bile acids, which may decrease the potential tumor promoter activity of secondary bile acids [364].

Furthermore, increased colonic acidification (pH below 6 to 6.5) may inhibit colonic bacterial enzyme 7 α-dehydroxylase, which degrades primary bile acids to secondary bile acids [365]. In

addition, decreased colonic pH increases the availability of calcium for binding to free bile acids and fatty acids [366]. In vitro and in vivo studies have shown that butyrate is the preferred energy substrate and stimulates cell proliferation in normal colonocytes [338,353], yet it suppresses proliferation of colon adenocarcinoma cells. This observed inconsistency has been termed the ‘‘butyrate paradox’’[359,362]. This discrepancy may be explained by differences between *in vitro* and *in vivo* environments, the timing of butyrate administration in relation to the stage of cancer development, the amount of butyrate administered, the source of butyrate (ie, different dietary fibers), and interaction with dietary fat [362]. SCFA enemas, especially butyrate, have also been used as a possible treatment for bowel inflammation, including diversion and ulcerative colitis. Roediger [367] demonstrated that colonocytes of individuals with active and quiescent ulcerative colitis have reduced butyrate oxidation compared with controls. Harig et al., [368] administered a SCFA enema solution of 60 mM of sodium acetate, 30 mM of sodium propionate, and 40 mM of sodium n-butyrate to 5 patients with diversion colitis for a period of 2 to 6 weeks. This study was the first to provide evidence that an absence or near absence of SCFAs often lead to rectosigmoid colitis, which suggested that a local nutrient deficiency led to an inflammatory state. The resupply of nutrients, either by surgical reanastomosis or SCFA irrigation, resulted in marked improvements by endoscopic appearance and histologic findings. However, another study using the same SCFA enema solution in 13 patients with diversion colitis resulted in no endoscopic or histologic changes after 2 weeks [369]. SCFA irrigation for the treatment of distal ulcerative colitis has produced inconsistent results [370], some showing it to be an effective treatment [371] and others not [372]. Possible explanations for the inconsistencies include type of SCFA used (mixture or butyrate alone), SCFA concentrations, frequency of administration, and duration of treatment. Mechanisms of action have been proposed to explain the use of SCFA irrigation as a possible treatment of bowel inflammation. These include a lack of luminal SCFAs (ie, a nutritional deficiency of colonic epithelium) and a block in the uptake or oxidation of SCFA by colonocytes [373,374], possibly because of a reduction in coenzyme A which is required for fatty acid oxidation [367]. It has been suggested that the latter may result from the production of sulfur-containing compounds by colonic microflora [375]. However, this block in uptake and oxidation may be overcome by ‘‘mass action’’; in other words, by raising SCFAs to higher than normal concentrations in the colonic lumen [374]. The use of SCFA irrigation as a treatment for colitis still remains inconclusive.

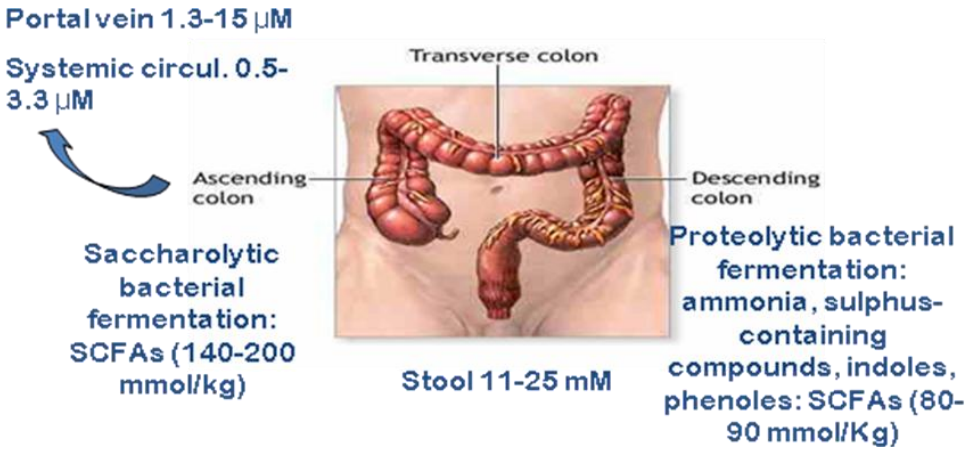


Fig. 4.2. Production and absorption of SCFAs along colon.

4.1 GPR43 and GPR41 as key receptors for short-chain fatty acids

G-protein-coupled receptors (GPCRs) are seven-transmembrane (7TM) receptors that mediate cellular responses to the majority of hormones and neurotransmitters, and are therefore attractive targets for drug discovery [376]. Free fatty acids (FFAs) have long been considered as key signaling molecules in numerous physiological and pathological processes. The recent identification of a family of GPCRs that bind FFAs has highlighted new potential mechanisms of action for FFAs in health and disease [377]. Among these FFAs receptors, GPR43 is present in a large variety of tissues, including adipose tissue, inflammatory cells, and gastrointestinal (GI) tract and it is activated by SCFAs [109,378]. The identification of these endogenous ligands of GPR43 has led the scientific community to propose a new appellation for GPR43, namely FFA2 or FFAR2 [377,379]. SCFAs bind GPR43 in the following rank order of potency: propionate > acetate=butyrate > valerate > formate [378,379]. Importantly, SCFAs also activate another receptor of the same family, GPR41, with propionate and butyrate being the most potent agonists [378,380]. Both receptors can couple to $G\alpha_{i/0}$ resulting in inhibition of the adenylate cyclase pathway, but only GPR43 is also able to couple to $G_{\alpha q}$, thus leading to activation of the phospholipase C (PLC) pathway and increased intracellular calcium levels [378,380]. GPR41 and GPR43 bind the same family of ligands (SCFAs), exhibit some overlapping expression, and partially share signaling pathways ($G\alpha_{i/0}$). Furthermore, both receptors represent potentially interesting targets for drug discovery.

GPR43 and GI tract functions

Since GPR43 is largely expressed throughout the gut, several authors have suggested that some effects of SCFAs could be GPR43-dependent. In 2006, Karaki et al. demonstrated that GPR43 was expressed in rat distal ileum and colon. Interestingly, peptide YY (PYY)-containing enteroendocrine L cells were immunoreactive for GPR43, whereas 5-hydroxytryptamine (5-HT) immunoreactive mast cells coexpressed GPR43 [381]. PYY is a satietogenic peptide that inhibits upper GI motility and SCFAs have been shown to induce its release in the blood [382]. Therefore, SCFAs might stimulate L cells to release PYY via GPR43 activation, thus slowing intestinal transit. GPR43 expression in these enteroendocrine L cells was observed likewise in the human colon [383]. SCFAs also exert physiological effects on colonic motility and secretion via 5-HT release

[384] and Karaki et al. proposed this might be attributable to the activation of GPR43 on 5-HT-containing mast cells [381]. The presence of GPR43 throughout the rat gut, with the lowest mRNA levels observed in the esophagus and stomach and the highest levels detected in the colon, was confirmed in another study [385].

Glucagon-like peptide 1 (GLP-1) is another gut hormone released by enteroendocrine L cells that is involved in the control of intestinal function and glucose metabolism [386]. SCFA infusion was shown to induce plasma GLP-1 release in animals and humans [387]. Interestingly, co-localization of GPR43 and GLP-1 in enteroendocrine L cells was demonstrated in both rat and human colon and terminal ileum [388]. In rodents, supplementation with fermentable carbohydrates increased GLP-1 production and the density of GPR43/GLP-1-positive enteroendocrine L cells in the proximal colon [388,389]. Therefore, a higher colonic production of SCFAs following dietary fiber fermentation increase GLP-1 secretion, *via* GPR43 activation in enteroendocrine L cells. This hypothesis has been recently confirmed by Tolhurst et al. [390]. The SCFA-triggered secretion of GLP-1 was almost completely abolished in primary colonic cultures from GPR43 KO mice but was also reduced, to a lesser extent, in mice lacking GPR41. GPR43-deficient mice had significantly reduced colonic GLP-1 protein content. Moreover, basal and glucose-stimulated levels of active GLP-1 were reduced in both GPR43 and GPR41 KO mice. These effects were associated with impaired glucose tolerance. Even if mice lacking GPR43 also exhibited decreased colonic expression of GPR41, a dominant role for GPR43 in SCFA-induced L cell activation was suggested based on the prevailing involvement of $G_{\alpha q}$ coupled pathways in this process (**Figure 4.3**) [390]. These results reveal again the difficulty to generate GPR43 KO mice without affecting GPR41, thus introducing uncertainties about their interpretation. Studies conducted in germ-free (GF) mice highlighted a potential link between gut microbiota and the expression of FFA receptors. The conventionalization (colonization by normal mouse microbiota) of GF mice increased adiposity and decreased the expression of GPR41 and GPR43 in the distal small intestine [111]. However, another study reported that GF mice exhibited decreased intestinal expression of GPR43, GPR41, PYY, and GLP-1 as compared with conventional mice. It is worth noting that the expression of GPR43 and GPR41 was differentially affected in GF mice, with a 10% and 70% decrease, respectively. This was associated with lower levels of circulating PYY [391]. Overall, these results suggest that gut microbiota can influence the intestinal expression of SCFA receptors and the secretion of gut peptides, but further studies are needed to elucidate the underlying mechanisms.

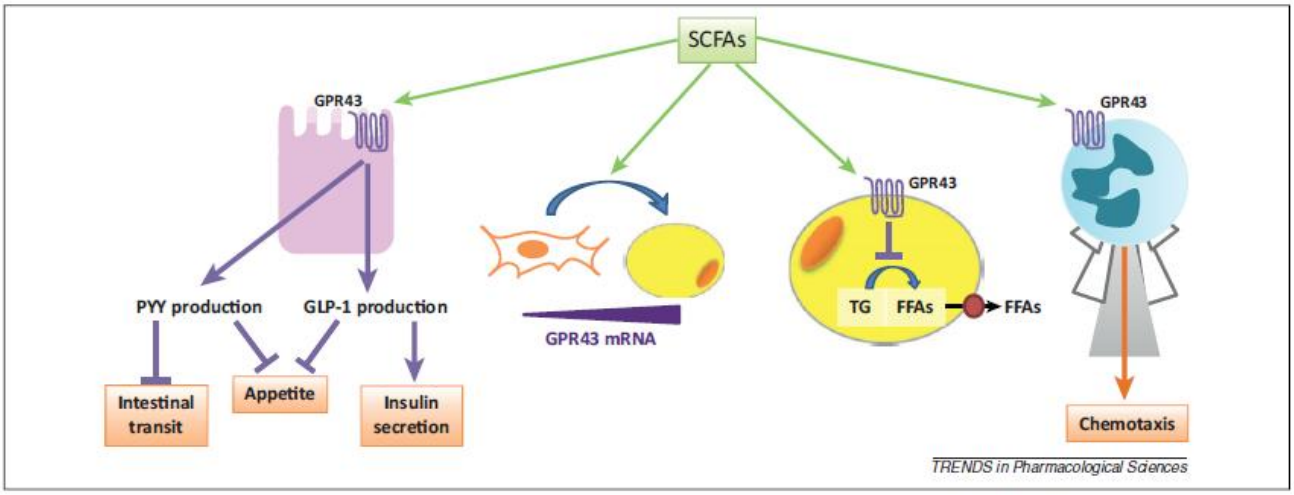


Fig. 4.3. Location and physiological functions of G-protein-coupled receptor 43 (GPR43). Studies in rodents have highlighted that SCFAs bind to GPR43 to exert several physiological actions. GPR43 activation on intestinal enteroendocrine cells (in pink) induces the production of PYY and GLP-1. PYY inhibits intestinal transit and appetite, whereas GLP-1 is anorexigenic and stimulates insulin secretion. In mice, GPR43 expression increases during adipogenesis and SCFAs stimulate adipocyte differentiation (fibroblast in orange). Through their binding to GPR43, SCFAs also inhibit lipolysis in mature adipocytes (in yellow). Finally, SCFAs induce chemotaxis of neutrophils (in blue) through GPR43 activation. Abbreviations: TG, triglycerides; FFAs, free fatty acids. *Image from : Bindels LB, Dewulf EM, Delzenne NM. GPR43/FFA2: physiopathological relevance and therapeutic prospects. Trends Pharmacol Sci. 2013 Apr;34(4):226-32. doi: 10.1016/j.tips.2013.02.002. Epub 2013 Mar 13.*

GPR43 and inflammation

SCFAs have long been known to modulate the production of pro- and anti-inflammatory mediators [392]. For instance, production of prostaglandin E2 is induced by SCFAs. This process can be inhibited by pertussis toxin, suggesting the involvement of a G-protein-mediated signalling [393]. The formal proof of GPR43 involvement in the management of inflammation was simultaneously provided by two research teams. They both established the contribution of GPR43 to the recruitment of immune cells [6,48], and this observation was further confirmed by others [394]. However, these studies showed divergent findings on the potential impact of GPR43 in inflammatory diseases. Maslowski et al., [109] demonstrated that stimulation of GPR43 by acetate allowed resolution of a colitis-related inflammatory response. GPR43 KO mice showed exacerbated or unresolved inflammation in cases of acute and chronic colitis, arthritis, and asthma. This could be related to increased immune cell recruitment [109]. By contrast, Sina et al. reported that, in an acute colitis model, GPR43 KO mice showed an increased mortality compared with control mice, despite reduced immune cell recruitment, decreased colonic inflammation, and attenuated colonic tissue damage. The increased mortality was attributed to septic complication. In a chronic colitis model, GPR43 deficiency led to reduced colonic inflammation, without any sign of sepsis and any lethality.

The authors pointed out the bipotential pathophysiological role of immune cells at the intestinal level, being a protective factor against acute bacterial transmigration, but having a detrimental role in chronic inflammatory responses [395]. Clearly, GPR43 is involved in the SCFA-induced neutrophil chemotaxis in mice (**Figure 4.3**) [109,394]. However, demonstrating GPR43 contribution to human neutrophil chemotaxis remains to be accomplished. Interestingly, GLPG0974, an orally available small GPR43 inhibitor from Galapagos with undisclosed structure, has been claimed to reduce neutrophil migration. GLPG0974 is currently being tested in a second Phase I study and results are expected in early 2013 (ClinicalTrials.gov; identifier: NCT01721980). The global role of GPR43 in inflammatory conditions needs to be clarified before ruling on the therapeutic potential of GPR43 in this context. The dualistic action of SCFAs, being anti-inflammatory while recruiting neutrophils, might be one of the keys to full understanding of how SCFAs and GPR43 manage inflammation. Importantly, a comparison of several studies highlights that the biological and molecular responses to SCFAs differ in the several type of immune cell [394]. This might be consistent with the various temporal roles of these cells in an inflammatory response [393]. Therefore, the different molecular pathways downstream of GPR43 remain to be elucidated. Finally, ITF prebiotic feeding can control inflammation in rodent models of colitis, obesity, diabetes, and leukemia [396,397]. It is possible to postulate that prebiotics, through their fermentation into SCFAs, might exert some of their anti-inflammatory effects in a GPR43-dependent manner, this hypotetesis need to be confirmed.

4.2 Effects of Butyrate at intestinal level

Effects on transepithelial ion transport

Potentially, SCFAs are absorbed by each intestinal segment, as demonstrated in animal models and human volunteers. The colonocytes absorb butyrate and other SCFAs through different mechanisms of apical membrane SCFA uptake, including non-ionic diffusion, SCFA/$HCO3^-$ exchange, and active transport by SCFA transporters. The transport proteins involved are monocarboxylate transporter isoform 1 (MCT1), which is coupled to a transmembrane H^+-gradient, and SLC5A8, which is Na^+-coupled co-transporter [398,399]. The absorption of these fatty acids has a significant impact on the absorption of NaCl and, generally, on the electrolyte balance [400]. In particular, butyrate is able to exert a powerful proabsorptive stimulus on intestinal NaCl transport and an anti-secretory effect towards Cl^- secretion. The powerful regulatory pro-absorptive/anti-secretory effects induced by butyrate on the transepithelial ion transport occurs through several mechanisms: (1) stimulation of NaCl absorption by the action of two coupled transport systems on the intestinal brush border: $Cl^-/HCO3^-$ and Na^+/H^+ and Cl^-/butyrate and Na^+/H^+; and (2) inhibition of Cl^- secretion by blocking the activity of the cotransporter Na^+-K^+-$2Cl^-$ (NKCC1) on the enterocyte basolateral membrane. *In vitro* studies have shown that butyrate has an inhibitory effect on Cl^- secretion induced by prostaglandin E2, cholera toxin, and phosphocholine. This effect is due to reduced production of intracellular cAMP secondary to the expression and regulation of adenylate cyclase [399]. Comparison studies showed that the pro-absorptive and anti-secretory effects of butyrate are significantly higher than those of all other SCFAs [400]. Clinical studies in children with acute diarrhea caused by *V. cholerae* showed a reduction in stool volume and a more rapid recovery in patients who received oral rehydration therapy in addition to resistant starch, a precursor of butyrate, in the diet [401]. These results were confirmed in other forms of infectious diarrhea in children and in animal models studies [402]. Moreover, butyrate therapy is beneficial in patients affected by Congenital Chloride Diarrhea (CLD) [403]. This rare genetic disease is caused by mutations in the gene encoding the solute-linked carrier family 26-member A3 (SLC26A3) protein, which acts as a plasma membrane anion exchanger for Cl^- and $HCO3^-$ [404]. The mechanism underlying this therapeutic effect could be related, at least in part, to stimulation of the Cl^-/butyrate exchanger activity [403]. It is also possible that butyrate could reduce mistrafficking or misfolding of the SLC26A3 protein, as demonstrated for other molecules involved in transepithelial

ion transport [405]. Alternatively, butyrate may enhance gene expression: the SLC26A3 gene contains a 290-bp region between residues -398 and -688 that is crucial for high-level transcriptional activation induced by butyrate. This may explain the variable response of patients affected by CLD to butyrate [406]. In fact, depending on the patient's genotype, mutations in the above-mentioned regulatory regions of the SLC26A3 gene could affect the gene transcription rate. It is also conceivable that other channels could be involved in the therapeutic effect of butyrate in CLD.

Effects on cell growth and differentiation

Several epidemiological studies support the role of dietary fiber in the protection against colorectal cancer [407,408]. Different mechanisms have been proposed for fiber's cancer preventive properties: reduction in transit time of the feces in the gut, which reduces exposure of the mucosa to luminal carcinogens; absorption of bile acids, biogenic amines, bacterial toxins, and production of butyrate. Most of the anticarcinogenic effects of butyrate are observed *in vitro* carcinoma cell lines. In these models, addition of butyrate leads to inhibition of proliferation, induction of apoptosis, or differentiation of tumor cells [409,410]. Butyrate's anticarcinogenic effects are in contrast with the effects of this compound in normal enterocytes. In fact, it has been shown that butyrate stimulates the physiological pattern of proliferation in the basal crypt in the colon, whereas it reduces the number and the size of aberrant crypt focus, which are the earliest detectable neoplastic lesions in the colon [411]. These contradictory patterns of butyrate represents the so called "butyrate paradox" [409]. An important mechanism by which butyrate causes biological effects in colon carcinoma cells is the hyperacetylation of histones by inhibiting histone deacetylase (HDAC). This compensates for an imbalance of histone acetylation, which can lead to transcriptional dysregulation and silencing of genes that are involved in the control of cell cycle progression, differentiation, apoptosis and cancer development [412,413]. In particular, in human colon cancer cell lines butyrate, acting as HDAC inhibitor, increases the p21 (WAF1) gene expression by selectively regulating the degree of acetylation of the gene-associated histones, and induces G1 cell cycle arrest [414]. A novel contributory mechanism to the chemopreventive effect of butyrate is the downregulation of the key apoptotic and angiogenesis regulator Neuropilin-1 (NRP-1), which has been shown to promote tumor cell migration and survival in colon cancer in response to vascular endothelial growth factor (VEGF) binding [415]. Several reports have shown that the apoptosis triggered by butyrate *in vitro* is associated with dysregulation of Bcl2 family proteins, especially

upregulation of BAK and downregulation of Bcl-xL [416], rather than cellular damage. A study by Thangaraju et al suggests a novel mode of action of butyrate in the colon involving GPR109A, a G-protein–coupled receptor for nicotinate [417], which recognises butyrate with low affinity. This receptor is expressed in the normal colon on the lumenfacing apical membrane of colonic epithelial cells, but is silenced in colon cancer *via* DNA methylation. Thangaraju et al., [417] showed that inhibition of DNA methylation in colon cancer cells induces GPR109A expression and that activation of the receptor causes tumor cell–specific apoptosis. Butyrate is an inhibitor of HDAC, but apoptosis induced by activation of GPR109A with its ligands in colon cancer cells does not involve inhibition of histone deacetylation. The primary changes in this apoptotic process include downregulation of Bcl-2, Bcl-xL, and cyclin D1 and upregulation of death receptor pathway. Moreover, a recent study suggested that the protective role of dietary fiber, and its breakdown product butyrate, against colorectal cancer could be determined by a modulation of canonical Wnt signaling, a pathway constitutively activated in the majority of colorectal cancers [418]. Butyrate is recognised for its potential to act on secondary chemoprevention, by slowing growth and activating apoptosis in colon cancer cells [359], but it can also act on primary chemoprevention. The mechanism proposed is the transcriptional upregulation of detoxifying enzymes, such as glutathione-S-transferases (GSTs). This modulation of genes may protect cells from genotoxic carcinogens, such as H_2O_2 and HNE [412].

Effects on inflammatory and oxidative status

Butyrate plays a role as an anti-inflammatory agent, primarily via inhibition of NF-κB activation in human colonic epithelial cells [419], which may result from the inhibition of HDAC. NF-κB regulates many cellular genes involved in early immune inflammatory responses, including IL-1β, TNF-α, IL-2, IL-6, IL-8, IL-12, iNOS, COX-2, intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), T cell receptor-α (TCR-α), and MHC class Ⅱ molecules [420]. The activity of NF-κB is frequently dysregulated in colon cancer [421] and in IBDs, such as ulcerative colitis and Crohn's disease [422]. In CD patients, butyrate decreases pro-inflammatory cytokine expression via inhibition of NF-κB activation and IκBα degradation [422]. The upregulation of PPAR-γ and the inhibition of IFN-γ signaling, are another two of butyrate's anti-inflammatory effects [423]. Butyrate can act on immune cells through GPR41 (or FFA3) and GPR43 (or FFA2), which are both expressed on immune cells, including polymorphonuclear cells, suggesting that butyrate might be involved in the activation of leucocytes [424]. The possible

immune-modulatory functions of SCFAs are highlighted by a recent study on GPR43 -/-mice. These mice exhibit aggravated inflammation, related to increased production of inflammatory mediators and increased immune cell recruitment [395]. Most clinical studies analyzing the effects of butyrate on inflammatory status focused on UC patients. Hallert et al. [425] instructed 22 patients with quiescent UC to add 60 g oat bran (corresponding to 20 g dietary fiber) to their daily diet. Four weeks of this treatment resulted in a significant increase of fecal butyrate concentration and in a significant improvement of abdominal symptoms. In a double blind, placebo-controlled multicenter trial, Vernia et al., [426] treated 51 patients with active distal UC with rectal enemas containing either 5-aminosalicylic acid (5-ASA) or 5-ASA plus sodium butyrate (80 mmol/L, twice a day). The combined treatment with topical 5-ASA plus sodium butyrate significantly improved the disease activity score more than 5-ASA alone. These and other intervention studies [427] suggested that the luminal administration of butyrate or stimulation of luminal butyrate production by the ingestion of dietary fiber results in an amelioration of the inflammation and symptoms in UC patients. Numerous studies have reported that butyrate metabolism is impaired in intestinal inflamed mucosa of patients with IBD. Recent data show that butyrate deficiency results from the reduction of butyrate uptake by the inflamed mucosa through downregulation of MCT1. The concomitant induction of the glucose transporter GLUT1 suggests that inflammation could induce a metabolic switch from butyrate to glucose oxidation. Butyrate transport deficiency is expected to have clinical consequences. Particularly, the reduction of the intracellular availability of butyrate in colonocytes may decrease its protective effects toward cancer in IBD patients [428]. Limited evidence from pre-clinical studies shows that oxidative stress in the colonic mucosa can be modulated by butyrate. Oxidative stress is involved in both inflammation [429] and the process of initiation and progression of carcinogenesis [430]. During oxidative stress there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense mechanisms, leading to a cascade of reactions in which lipids, proteins, and/or DNA may get damaged. In healthy humans, it has been demonstrated that locally administered butyrate in physiological concentrations increased the antioxidant GSH and possibly decreased ROS production, as indicated by a decreased uric acid production [431]. As the human colon is continuously exposed to a variety of toxic stimuli, enhanced butyrate production in the colon could result in an enhanced resistance against toxic stimuli, thus improving the barrier function. This might be relevant for the treatment of gastrointestinal disorders, such as post-infectious irritable bowel syndrome (IBS), microscopic colitis, IBDs, and diversion colitis.

Butyrate and intestinal epithelial permeability

Intestinal epithelial permeability has been widely studied as an important parameter of the intestinal defence barrier. Under normal conditions, the epithelium provides a highly selective barrier that prevents the passage of toxic and proinflammatory molecules from the external milieu into the submucosa and systemic circulation. Macromolecules pass the epithelial barrier mainly *via* the paracellular route for which tight junctions are the rate-limiting structures [432]. Increased permeability, indicating impaired epithelial barrier function, is thought to be involved in the pathophysiology of several gastrointestinal inflammatory diseases, but can either be a cause or a consequence of inflammation [433]. Several studies have assessed the effects of butyrate on intestinal permeability in vitro as well as ex vivo. At low concentrations, butyrate (up to 2 mM) induces a concentration-dependent reversible decrease in permeability in a Caco-2 and HT-29 cell lines [434,435]. This decrease in permeability may be related to the butyrate associated increased expression of tight junction proteins observed in different cultured cell lines, but this effect was shown to be cell type dependent [436]. At higher concentrations (8 mM), however, butyrate increased the permeability in a Caco-2 cell line [435]. An *ex vivo* study, using adult rat distal colon mucosa mounted in an Ussing chamber, demonstrated that acute exposure to butyrate at a concentration of 10 mM, but not 1 or 5 mM increased paracellular permeability in rat colon [437]. This has also been demonstrated in rats fed a diet-containing fermentable FOS. The rapid bacterial fermentation of FOS led to accumulation of high concentrations of SCFAs that increased intestinal permeability and was associated with increased translocation of *Salmonella* [438]. However, in humans, daily FOS supplementation of 20 g did not increase intestinal permeability [439]. It can be concluded that the effect of butyrate on intestinal permeability depends on its concentration and on the model system or species used. The effects of butyrate at different concentrations remain to be evaluated in the human *in vivo* situation.

Effects on visceral perception and intestinal motility

Little is known about the environmental and nutritional regulation of the enteric nervous system (ENS), which controls gastrointestinal motility. Butyrate regulates colonic mucosa homeostasis and can modulate neuronal excitability. Soret et al., [440] investigated the effects of butyrate on the ENS and colonic motility, and showed, *in vivo* and *in vitro*, that butyrate significantly increased the proportion of choline acetyltransferase (ChAT), but not neuronal nitric

oxide synthase (nNOS) immunoreactive myenteric neurons. Butyrate increases the cholinergic-mediated colonic circular muscle contractile response *ex vivo*. The authors suggest that butyrate might be used, along with nutritional approaches, to treat various gastrointestinal motility disorders associated with inhibition of colonic transit. A recent study by Vanhoutvin et al., [441] shows that intraluminal administration of a physiologically relevant dose (50 to 100 mmol/L-) of butyrate into the distal colon increases compliance and decreases pain, urge, and discomfort measured with a rectal barostat procedure in healthy subjects. This study suggests a potential beneficial effect of butyrate in disorders that are associated with visceral hypersensitivity, such as IBS and infantile colics, and provides a basis for future trials with dietary modulation resulting in intracolonic butyrate production in both healthy and IBS subjects. The decrease in visceral perception induced by butyrate treatment could be due to an increased 5-HT release, as previously suggested by others [442]. Another possible mechanism by which butyrate could affect visceral perception is the previous reported inhibition of histone deacetylase. In fact, Chen et al., [443] showed that these inhibitors induce microglyal apoptosis and attenuate inflammation-induced neurotoxicity in rats, which may affect visceral perception. Butyrate has been reported to induce enhancement of colonic motility via the release of 5-HT [444]. In functional studies, butyrate and propionate induced phasic and tonic contractions in rat colonic circular muscle. The dose-dependent contractile effect occurred only when SCFAs were applied on the mucosal side and disappeared in mucosal free preparations, suggesting the presence of sensory mechanisms near the epithelium [384].

Effects on non-specific intestinal defense mechanisms

Besides the effects of butyrate on carcinogenesis, inflammation and oxidative stress, butyrate has been shown to affect several components of the colonic defence barrier leading to enhanced protection against luminal antigens. One important component of this barrier is the mucous layer covering the epithelial lining consisting of mainly mucin glycoproteins and trefoil factors (ITF or TFF3). Mucin glycoproteins are classified into neutral and acidic subtypes and the latter category further includes sulfomucins and sialomucins. Sulphated mucins are generally considered to be more resistant to bacterial degradation [445]. Several epithelial mucin (MUC) genes have been identified in humans, of which MUC2 is predominantly expressed in the human colon [446]. Alterations in goblet cell function, composition and thickness of the intestinal mucous layer have been found in several intestinal disorders. For example, a reduced mucous thickness and a decreased MUC2 production have been reported in UC patients [447]. *In vitro* studies, butyrate

increased the MUC2 gene expression in specific cell lines [448,449]. In addition, 0.1–1 mM butyrate administered to human colonic biopsy specimens *ex vivo* stimulated mucin synthesis [450]. Luminal butyrate administration of 5 mM, but not 100 mM, increased mucous secretion in an isolated perfused rat colon [451]. In another rat study, caecal and faecal SCFA concentrations were found to correlate with mucous thickness. In humans, effects of butyrate alone on mucous synthesis, thickness of the mucous layer and MUC expression *in vivo* have not been reported. The effects of a number of fermentable dietary fibres on the mucous layer have been studied with varying results. For example, resistant starch increased the number of acidic mucins, but did not affect the number of goblet cells in rats [452]. In contrast, FOS increased the number of goblet cells in piglets [453]. In a human intervention study with patients with an ileo-anal pouch, inulin supplementation did not alter MUC2 expression or the ratio between sulfomucins and sialomucins [454]. Trefoil factors are mucin-associated peptides that contribute to the viscoelastic properties of the mucous layer. TFFs are thought to reduce the recruitment of inflammatory cells and to be involved in the maintenance and repair of the intestinal mucosa, although the exact mechanism for this effect is not yet known [455]. Intestinal trefoil factor is almost exclusively secreted by the intestinal goblet cells [456]. In a rat TNBS model of colitis, TFF3 expression was decreased during active disease, and intracolonic administration of butyrate increased TFF3 expression [457]. However, butyrate inhibited the expression of TFF3 in colon cancer cell lines and in colonic tissue of newborn rats [458]. Other components of the colonic defence barrier that are involved in the maintenance of the colonic barrier, which may be influenced by butyrate are transglutaminase, antimicrobial peptides and heat shock proteins (HSPs). The enzyme transglutaminase is actively involved in intestinal mucosal healing and correlates with the severity of inflammation in UC [459]. In a rat model of colitis, butyrate restored the colonic transglutaminase levels [460]. Antimicrobial peptides such as cathelicidin (LL-37) and defensins, protect the gastrointestinal mucosa against the invasion and adherence of bacteria and thereby prevent infection [461,462]. Several *in vitro* studies have shown that butyrate upregulates the expression of LL-37 in different colon epithelial cell lines as well as in freshly isolated colorectal epithelial cells [463]. HSPs confer protection against inflammation by suppressing the production of inflammatory modulators [464,465]. Butyrate induced the expression of HSP70 and HSP25 in Caco-2 cells [465] and in rats [452,464]. However, in a study in rats with DSS induced colitis, butyrate inhibited HSP70 expression. This was related to protection against the decrease in cell viability, increase in mucosal permeability and neutrophil infiltration in DSS colitis. It was concluded that the induction of heat shock response has a protective effect before an injury, whereas activation of heat shock response leads to cytotoxic effects after a proinflammatory

stimulus [466]. In addition, there is evidence from in vitro studies with human colon cancer cell lines that butyrate is involved in repair after mucosal damage through an increase in the rate of cell migration. Efficient repair of superficial injuries and mucosal ulcers is important in maintaining and re-establishing the epithelial barrier [467]. In conclusion, there are several lines of evidence suggesting that butyrate reinforces the colonic defence barrier by affecting several components of this barrier, such as the promotion of epithelial migration and the induction of mucins, TFF, transglutaminase activity, antimicrobial peptides and HSPs. However, most of these effects still have to be confirmed in the human studies. The effects of butyrate at intestinal levels are resumed in schematic **figure 4.4.**

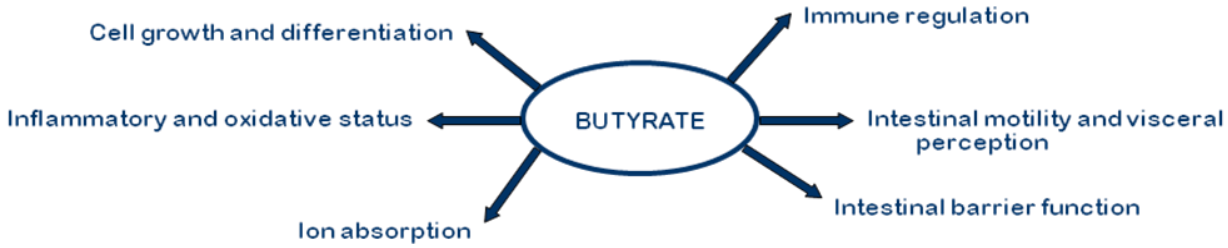


Fig. 4.4. Effects of butyrate at intestinal level.

4.3 Extraintestinal Effects of Butyrate

Hemoglobinopathies

Clinical trials in patients with sickle cell disease and β-thalassemia confirmed the ability of butyrate to increase fetal hemoglobin (HbF) production [468,469]. Butyrate is an inducer of HbF through an epigenetic regulation of fetal globin gene expression via HDAC inhibition, resulting in global histone hyperacetylation, including nucleosomes at the γ-globin promoters [470]. Other experiments have shown that butyrate can cause a rapid increase in the association of γ-globin mRNA with ribosomes [471]. Other authors have demonstrated activation of p38 mitogen activated protein kinases and cyclic nucleotide signaling pathways in association with butyrate induction of HbF [472]. Taken together, these studies suggest that global histone hyperacetylation induced by HDAC inhibition is not the unique mechanism underlying butyrate stimulation of HbF.

Genetic metabolic diseases

Sodium phenylbutyrate 4 (4-PBA) was approved by the Food and Drug Administration (FDA) for use in patients with urea cycle enzyme deficiency, in which it acts as a scavenger of ammonia. Indeed, 4-PBA is oxidized to phenylacetate, which binds to glutamine and determines the urinary excretion. In patients with ornithine transcarbamylase deficiency, the use of 4-PBA allows for better metabolic control and increased intake of natural protein in the diet [473]. The possible use of butyrate in the treatment of X-linked Adrenoleukodystrophy (X-ALD), a disorder of peroxisomes characterized by altered metabolism and accumulation of very long chain fatty acids, has also been studied. Sodium phenylbutyrate 4 induces, *in vitro* on fibroblasts from patients with X-ALD and *in vivo* in X-ALD knockout mice, an increase in β-oxidation of very long chain fatty acids and peroxisome proliferation [474].

Hypercholesterolemia

Under normal lipidemic conditions, the liver is the most important site of cholesterol biosynthesis, followed by the intestine. Biosynthesis in the liver and intestine account for about 15% and 10%, respectively, of the total amount of cholesterol biosynthesis each day [475]. In hypercholesterolemia, when cholesterol biosynthesis is suppressed in most organs by fasting, the intestine becomes the major site of cholesterol biosynthesis, and its contribution can increase up to 50%. Importantly, recent evidence shows that the global effect of butyrate is to downregulate the expression of nine key genes involved in intestinal cholesterol biosynthesis, potentially inhibiting this pathway [476].

Obesity and insulin resistance

Dietary supplementation with butyrate can prevent and treat diet-induced obesity and insulin resistance in mouse models. After a 5-wk treatment with butyrate, obese mice lost 10.2% of their original body weight. Consistent with the change in body weight, fat content was reduced by 10%. Furthermore, fasting glucose was reduced by 30%, insulin resistance was reduced by 50%, and intraperitoneal insulin tolerance was improved significantly by butyrate. The mechanism of butyrate action is related to promotion of energy expenditure and induction of mitochondrial function. Stimulation of peroxisome proliferator-activated receptor (PPAR) coactivator (PGC-1α) activity has been suggested as the molecular mechanism of butyrate. Activation of AMPK and inhibition of histone deacetylases may contribute to the PGC-1α regulation. These data suggest that butyrate may have potential application in the prevention and treatment of metabolic syndrome in humans [477].

Butyrate and satiety

It has been hypothesized that SCFAs produced in the large intestine also can influence upper gut motility and satiety [478]. Endocrine L-cells present in large concentrations in the colonic mucosa secrete peptides such as GLP-1, peptide YY and oxyntomodulin, which are involved in appetite regulation and satiety [479]. In several animal studies using fermentable carbohydrates such as inulin [479], lactitol [480] and FOS [481], an increased satiety, decreased weight gain and increased endogenous production of GLP-1 and/or PYY were reported. In humans, FOS increased satiety [482] and increased plasma GLP-1 concentrations [483]. However, lactitol did not affect

plasma concentrations of this gut peptide [480]. The increased satiety is possibly promoted through the production of SCFAs. This is supported by a number of studies. Butyrate increased the expression of PYY and proglucagon *in vitro* in rat epithelial cells [484] and increased PYY release, but not that of GLP-1, in the isolated colon of rats [485] and rabbits [486]. In addition, colonic SCFA infusion in rats stimulated PYY release [487]. However, colonic infusion with SCFAs in humans did not increase plasma levels of either PYY or GLP-1 [488]. Activation of the SCFA receptor GPR43 expressed in endocrine L-cells may play a role in this effect on satiety [383]. There is increasing evidence that the effect of fermentable dietary fibre on satiety is mediated through the colonic production of SCFAs. However, most evidence originates from rat studies, while again human evidence remains limited. In **figure 4.5** the main intestinal SCFA receptors and transporters are shown.

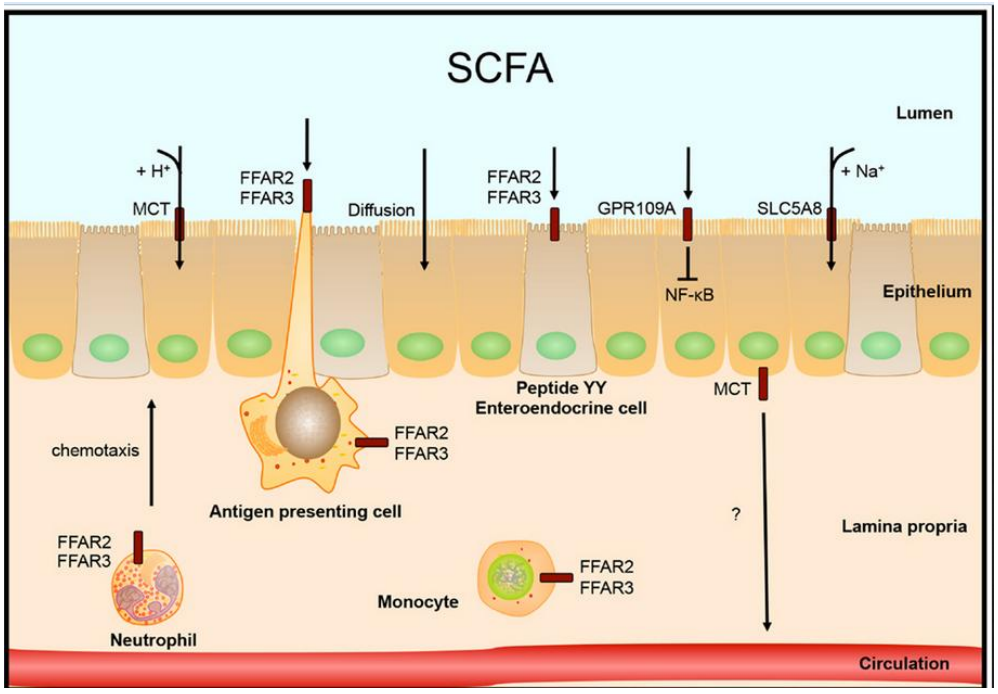


Fig. 4.5. Intestinal SCFA receptors and transporters. SCFAs are taken up by the epithelial cells by diffusion, H^+ coupled transport by monocarboxylate transporters (MCT) or by Na^+ coupled transport by SLC5A8. Other receptors that are activated by SCFA are localized on colonocytes, peptide YY expressing enteroendocrine cells, or different immune cells. Receptor FFAR2 is involved in neutrophil chemotaxis toward sources of SCFA. *Image from: R. Schilderink, C. Verseijden and W. J. de Jonge. Dietary inhibitors of histone deacetylases in intestinal immunity and homeostasis. Front. Immunol., 01 August 2013 doi: 10.3389/fimmu.2013.00226.*

Adverse effects of butyrate

In contrast to the wide range of positive effects of butyrate on the intestinal mucosa, a small number of studies have also shown some adverse effects. Two rat studies revealed that rectal administration of butyrate (8–1000 mM), dose dependently increased colonic visceral sensitivity [489]. However, these effects have not yet been reported in humans. In faeces of weaning children, low butyrate concentrations have been measured [490]. It has been hypothesized that overproduction or accumulation of SCFAs may be toxic to the intestinal mucosa of premature infants and might play a role in the pathogenesis of neonatal necrotizing enterocolitis. It has been demonstrated that the severity of mucosal injury to butyrate, measured in newborn rats, was dose dependent and also depended on the maturation of the intestine [458,491]. It remains to be established whether luminal butyrate in premature infants can increase towards levels that are toxic for the intestinal mucosa [491]. In addition, as mentioned before, increased permeability and Salmonella translocation has been found after FOS supplementation in a study with rats, which may be the result of SCFA accumulation [438]. However, this was not confirmed in the humans [439].

EXPERIMENTAL SECTION

CHAPTER 5: EFFECTS OF A LACTOBACILLUS PARACASEI B21060 BASED SYNBIOTIC ON STEATOSIS, INSULIN SIGNALING AND TOLL-LIKE RECEPTOR EXPRESSION IN RATS FED A HIGH-FAT DIET

5.1 Introduction

Non-alcoholic fatty liver disease is an increasingly recognized clinical condition characterized by insulin resistance, hepatic steatosis and frequently type 2 diabetes (T2DM). The pathophysiology of NAFLD is still not completely defined. Tilg and Moschen have proposed a "parallel hits" hypothesis on the evolution of inflammation in NAFLD [29], as opposed to the so called "two hits" previously suggested for the development of nonalcoholic steatohepatitis (NASH) [28]. This new model suggests that different hits may act in parallel, and that gut- or adipose tissue derived factors may have a key role in the onset of liver inflammation. The cytokines tumor necrosis factor α (TNF-α) and interleukin (IL)-6 represent a link between IR and liver inflammatory process, activating several mechanisms involved in hepatocyte apoptosis and inhibition of insulin signaling [93,492]. Evidence suggest the modulation of gut microflora as potential target for the prevention and treatment of NAFLD [493,494]. Probiotics are live microbial that have beneficial effects on human health and disease modulating intestinal microbiota composition and function, improving epithelial barrier function, and reducing inflammation [495]. Immune and epithelial cells can discriminate among different microbial species through the activation of TLRs [496]. We have recently obtained experimental and clinical evidences that selected probiotics, could be effective against NAFLD [70,497]. The effects of probiotics are clearly related to specific strains and dosage [498]. It has been reported that some lactic acid bacteria affect the progression of diabetes mellitus [259,396]. These studies show that ingestion of determined lactic acid bacteria prevents or delays the disease onset in various experimental models of diabetes, induced by a chemical or by diet, or genetically modified animals (db/db) [499]. A variety of *in vitro* experiments and *in vivo* studies provided experimental evidence to support the probiotic roles in lowering serum cholesterol and ameliorating lipid profiles [500]. It has been demonstrated that *L. paracasei B21026*, alone or in combination with prebiotics, is effective to limit infectious diseases and to regulate immune system [246,501]. A recent study has highlighted the striking difference among species and strains of

lactobacilli such as *L. plantarum NCIMB8826, L. rhamnosus GG* and *L. paracasei B21060* in modulating immune and inflammatory response [240]. This latter strain of lactobacillus, the most active, was isolated from the feces of breast-fed babies and its non-occasional presence in the physiological intestinal microflora was established by genetic identification methods [502]. Based on these findings, it seemed of great interest to assess the influence of a synbiotic preparation containing *Lactobacillus paracasei B21060* on liver damage and glucose homeostasis using an animal model of NAFLD. This synbiotic is commercially available in Europe as a formulation containing prebiotics (arabinogalactan and fructo-oligosaccharide) that are able to improve probiotic strain survival [503]. Here, we have demonstrated that this synbiotic could limit inflammatory liver damage and insulin signalling impairment by restoring intestinal permeability and, thus, preventing the imbalance of TLR pattern in a model of IR and steatosis in young rats.

5.2 Materials and methods

Ethics Statement

This study was carried out in strict accordance with the Institutional Guidelines and complied with the Italian D.L. no. 116 of January 27, 1992 of Ministero della Salute and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC). All animal procedures reported herein were approved by the Institutional Committee on the Ethics of Animal Experiments (CSV) of the University of Naples "Federico II" and by the Ministero della Salute under protocol no. 2008-0099793. Prior to sample collection, animals were euthanized by an intraperitoneal injection of a cocktail of ketamine/xylazine, followed by cervical dislocation to minimize pain. All efforts were made to minimize animal suffering.

Diets and synbiotic

High fat diet (HFD) provided in pellet with 58% of energy derived from fats, 18% from proteins, and 24% from carbohydrates (5.56 kcal/g) was purchased from Laboratorio Dottori Piccioni (Gessate, Milan, Italy). The composition of this diet has been previously described [255]. The control standard (STD) pellet diet had 15% of energy from fats, 22% from proteins, and 63% from carbohydrates (3.30 kcal/g). The synbiotic formulation containing viable lyophilized *L. paracasei B21060* mixed with prebiotics fructooligosaccharides and arabinogalactan (Flortec, Bracco, Milan, Italy) was available as powder and dispensed in 6 g bag containing about 2.5×10^9 CFU of the bacteria.

Animal model and experimental design

After weaning, young male Sprague–Dawley rats (113.5±1.1 g; Harlan, Corezzano, Italy) were randomly allocated in 3 groups (at least n=8) as follows: (1) control group, receiving STD and vehicle (tap water); (2) HFD-fed group, receiving vehicle; and (3) HFD-fed group, receiving the synbiotic by gavage once daily [HFD+SYN; *L. paracasei B21060* 2.5×10^7 bacteria/100 g body weight (bw); fructo-oligosaccharides 7 mg/100 g bw, and arabinogalactan 5 mg/100 g bw]. The

synbiotic treatment started together with the HFD and continued for 6 weeks. The HFD, administered for a long period of time (up to 6 months), creates a nutritional model of IR and NASH in non-genetically modified animals [256]. In our experiments, we administered HFD in young rats for a shorter period of time (6 weeks) to induce the early events of NAFLD due to fat overnutrition in young animals, excluding age and gender influences. Blood samples from animals were collected by cardiac puncture and serum obtained. Liver and white adipose tissue were excised and immediately frozen.

Histological analysis of liver tissue and transaminase levels

Liver sections were stained with hematoxylin and eosin or Oil Red O. Steatosis was graded on a scale of 0 (absence of steatosis), 1 (mild), 2 (moderate) and 3 (extensive). Alanine amino transferase (ALT) and aspartate amino transferase (AST) were measured in serum samples by standard automated procedures, according to manufacturer's protocols (AST Flex reagent cartridge, ALT Flex reagent cartridge; Dade Behring, Newark, DE, USA). Blood nonesterified fatty acids (NEFA) were determined as previously described [504].

Oral glucose tolerance test and insulin resistance assessment

At fifth week of treatment, fasted rats received glucose (2g/kg; per os) and glycaemia was measured at 0, 30, 60, 90 and 120 min after glucose administration. The area under the curve (AUC) was calculated from time zero, as the integrated and cumulative measure of glycemia up to 120 min for all animals. Glucose and insulin levels were measured by the glucometer One Touch UltraSmart (Lifescan, Milpitas, CA, USA) and by rat insulin radioimmunoassay kit (Millipore Corporation, Billerica, MA, USA), respectively. As index of insulin resistance, homeostasis model assessment (HOMA) was calculated, using the formula [HOMA=fasting glucose (mmol/L)×fasting insulin (μU/ml)/22.5].

Western blotting

Liver and visceral white adipose tissues were homogenized and total protein lysates were subjected to SDS-PAGE. Blots were probed with anti-suppressor of cytokine signaling 3 (SOCS3, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or antiperoxisome proliferator-activated

receptor α (PPAR-α; Santa Cruz Biotechnology), or anti-TLR4 (Imgenex, San Diego, CA, USA), or anti-PPAR-γ (Novus Biologicals, Littleton, CO, USA), or anti-glucose transporter4 (GLUT4, Santa Cruz Biotechnology). To evaluate NF-κB activation, IκB-α (Santa Cruz Biotechnology) and NF-κB p50 (Santa Cruz Biotechnology) were measured in liver cytosolic or nuclear extracts, respectively. Western blot for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma-Aldrich; Milan Italy) or lamin A (Chemicon, Temecula, CA, USA) was performed to ensure equal sample loading.

Immunoprecipitation

Immunoprecipitation of insulin receptor substrate (IRS)-1 was performed incubating 1.5 mg of liver lysate with 2 μg of an antibody against total IRS-1 (Santa Cruz Biotechnology). The immunoprecipitates were subjected to SDS-PAGE, and immunoblotted with an antibody against total IRS-1 or phospho-IRS-1Ser^{307} (1:1000, Cell Signaling Technology, Danvers, MA, USA).

Real-time semi-quantitative polymerase chain reaction (PCR)

Total RNA, isolated from liver, colon and visceral adipose tissue, was extracted using TRIzol Reagent (Invitrogen Biotechnologies), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 μg total RNA. PCRs were performed with an ABIPrism HT7900 fast Real-time PCR System instrument and software (Applied Biosystem). The primer sequences are reported in **Table 5.1**. The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample contained 1–100 ng cDNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/L of each primer (EUROGENTEC Explera s.r.l, Ancona, Italy) in a final volume of 25 μl. The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

Table 5.1. Real-Time PCR Primer Sequences

Target gene	Forward primer (5'→3')	Reverse primer (3'→5')	Accession Number
Adiponectin	AATCCTGCCCAGTCATGAAG	TCTCCAGGAGTGCCATCTCT	NM_144744
CD14	GTGCTCCTGCCCAGTGAAAGAT	GATCTGTCTGACAACCCTGAGT	AF_087943
FGF21	AGATCAGGGAGGATGGAACA	ATCAAAGTGAGGCGATCCATA	NM_130752.1
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	NM_017008 XM_216453
IL-6	ACAAGTGGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC	NM_012589
MyD88	TGGCCTTGTTAGACCGTGA	AAGTATTTCTGGCAGTCCTCCTC	NM_198130.1
Occludin	TTGGGAGCCTTGACATCTTGTTC	TCCGCCATACATGTCATTGCTTGGTG	NM_031329.2
RPL19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT	NM_009078.2
TLR2	GTACGCAGTGAGTGGTGCAAGT	TGGCCGCGTCATTGTTCTC	NM_198769 XM_227315
TLR4	CTACCTCGAGTGGGAGGACA	ATGGGTTTTAGGCGCAGAGTT	NM_019178
TLR9	ATGGCCTGGTAGACTGCAACT	TTGGCGATCAAGGAAAGGCT	NM_198131
TNF-α	CATCTTCTCAAAACTCGAGTGACAA	TGGGAGTAGATAAGGTACAGCCC	NM_012675
ZO-1	CCATCTTTGGACCGATTGCTG	TAATGCCCGAGCTCCGATG	NM_001106266 .1

Measurement of gut permeability in vivo

In another experiment, after 6 weeks on HFD, rats were fasted for 6 h and then gavaged with 4,000 kDa FITC-labeled dextran diluted in water (TdB Consultancy AB, Uppsala, Sweden) (500 mg/kg, 125 mg/ml). After 2 h, blood (500 µl) was collected from intracardiac puncture and centrifuged (3000 rpm for 15 min at RT), and FITC-dextran concentration in plasma was determined by spectrophotometry (excitation wavelength 485 nm; emission wavelength 535 nm; HTS-7000 Plus-plate-reader; Perkin Elmer, Wellesley, MA, USA), as previously described [505].

Immunofluorescence analysis of occludin and zonula occludens (ZO)-1

Colon segments were immediately removed, washed with phosphate-buffered saline (PBS), mounted in embedding medium (Pelco Cryo-Z-T, Ted Pella inc, Redding, California), and stored at

−80°C until use. Cryosections (7 μm) were fixed in formaldehyde 2%+PBS at RT for 10 min for occludin or in methanol for 10 min at RT for ZO-1. Non-specific background was blocked by incubation with normal goat serum in PBS and 0.1% Triton X-100. Sections were incubated for 2h with rabbit anti-occludin (1:50 for occludin, Santa Cruz Biotechnology) or rabbit anti-ZO-1 (1:100 for ZO-1; Invitrogen, Camarillo, CA, USA). Sections were probed with goat anti-rabbit Alexa Fluor 488 antibodies (1:200, Invitrogen). Slides were mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA), and visualized on a fluorescence microscope using a 640 objective, and images were stored digitally with Leica software. Two negative controls were used: slides incubated with or without primary antibody. All the staining were performed in duplicate in non-serial distant sections, and analyzed in a double-blind manner by two different investigators.

Semi-quantitative and qualitative assessment of Enterobacteriales order and Escherichia coli species by sequence analysis of the microbial 16S rRNA gene

Semi-quantitative PCR was performed to investigate modifications in Gram negative bacteria relative amount in animals receiving HFD alone or in combination with the synbiotic. For microbial content, DNA was extracted from colon tissue by the NucleoSpin Tissue (Macherey-Nagel, Düren, Germany). Group-specific primers based on 16S rRNA gene sequences PCR assay were forward Enterobacteriales order, CCTTGGTGATTGACGTTACTCGCA; reverse Enterobacteriales order, CCACGCTTTCGCACCTGAGC;forward Escherichia coli CATGCAGTCGAACGGTAACAGGA; reverse Escherichia coli, CTGGCACGGAGTTAGCCGGTG (Eurofins MWG Operon; Huntsville, AL, USA). The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample contained 50 ng DNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/l of each primer) in a final volume of 25 μl. PCR was performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories) The relative amount of 16S rRNA was normalized to RPL19 rRNA levels as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data are presented as mean±S.E.M. Statistical analysis was performed by analysis of variance test for multiple comparisons followed by Bonferroni's test, using Graph- Pad Prism (Graph-Pad software, San Diego, CA, USA). Statistical significance was set at $P<0.05$.

5.3 Results

Effects of the synbiotic on liver steatosis and damage

Liver sections from HFD-fed rats demonstrated hepatic damage compared to control animals. As shown in **Fig. 5.1A**, foci of inflammatory cell infiltration and hepatocyte necrosis or apoptosis appeared throughout the lobule. HFD-fed rats showed microvesicular steatosis of grade 2 (**Fig 5.1B**). In the animals treated with synbiotic the severity of steatosis was reduced at grade 1, with a microvescicular pattern of lipids accumulation mainly in perivenular and periportal region. Scattered inflammation and occasionally apoptotic nuclei were observed, showing that treatment prevents the inflammation induced by HFD. Accordingly, the increase in AST, ALT (**Fig. 5.1C and D**) and NEFA (**Fig. 5.1E**) were reduced by the synbiotic. Weight gain of HFD fed animals did not significantly change among groups after 5weeks (STD 209.2±7.2, HFD 225.0±6.6, and HFD+synbiotic 213.3±9.3 g), in accordance, at this experimental time, also fat mass did not vary among groups (STD 29.05±2.50, HFD 31.78±1.26, and HFD+synbiotic 30.30±2.88 g). Moreover, food intake, expressed as grams of food taken daily, did not differ between rats fed the HFD treated with vehicle (15.45±0.58 g/day/rat) or treated with synbiotic (15.45±0.82 g/day/rat).

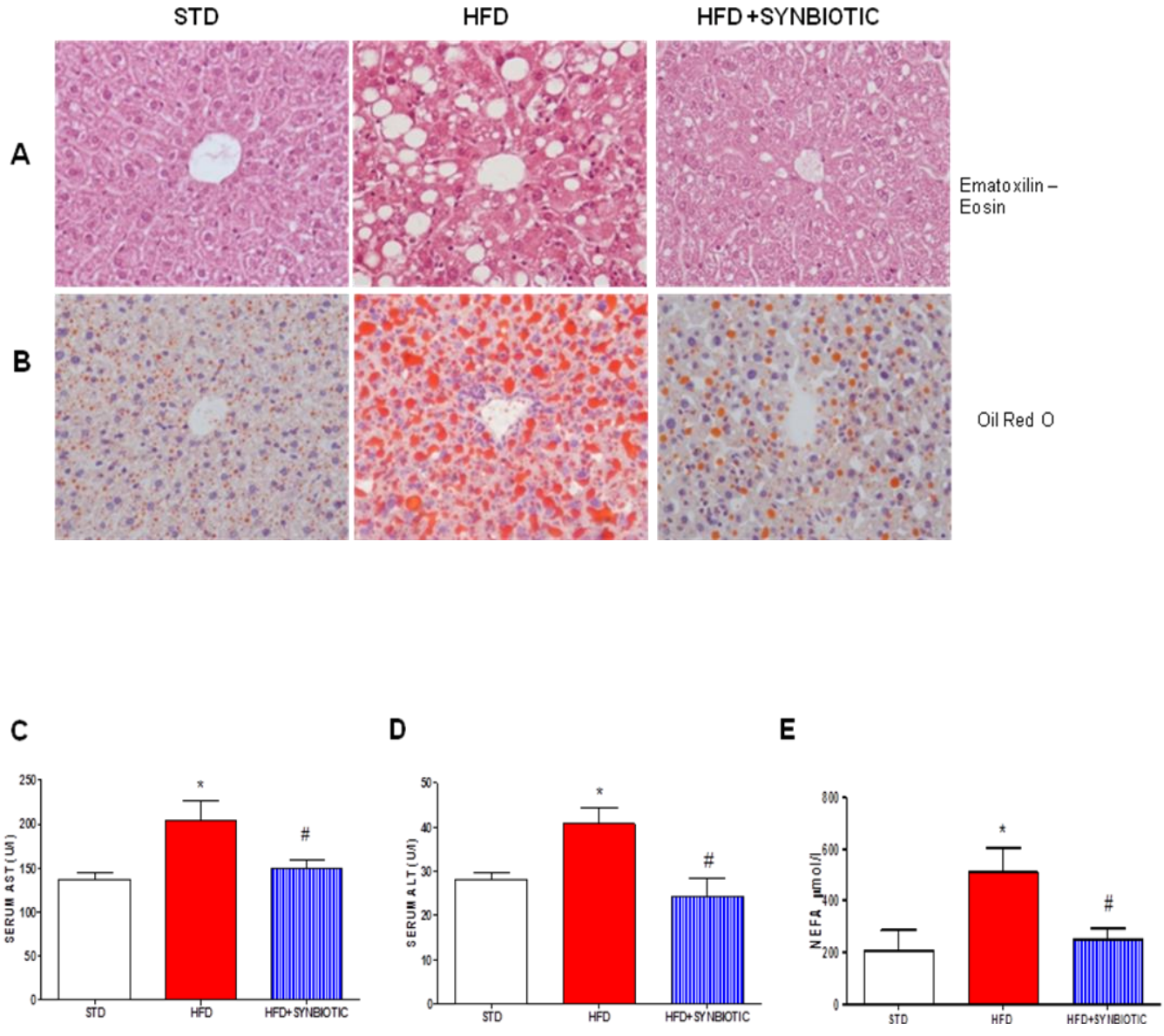


Fig. 5.1. The synbiotic effects on liver damage in HFD-fed rats. Paraffin-embedded sections of the liver (n=4 each group) were stained with hematoxylin-eosin (A) or oil red O (B). Micrographs in both panels are representative pictures with magnification 400×. Circulating AST (C), ALT (D), and NEFA (E) were measured (n=8, each group). (* P<0.05 *vs.* STD; # P<0.05 *vs.* HFD).

Effect of the synbiotic on glucose homeostatasis

The synbiotic administration caused a significant reduction of glycemia 90 min after glucose load (**Fig. 5.2A**). A marked and significant increase of AUC values was shown in HFD group (**Fig. 5.2B**), and this effect resulted significantly inhibited by synbiotic. As shown in **Fig 5.2C and D**, the increase in serum glucose and insulin levels induced by HFD was prevented in rats receiving the

synbiotic. Accordingly, IR assessed by the HOMA index was reduced (**Fig. 5.2E**). No significant difference in body weight was observed among all groups.

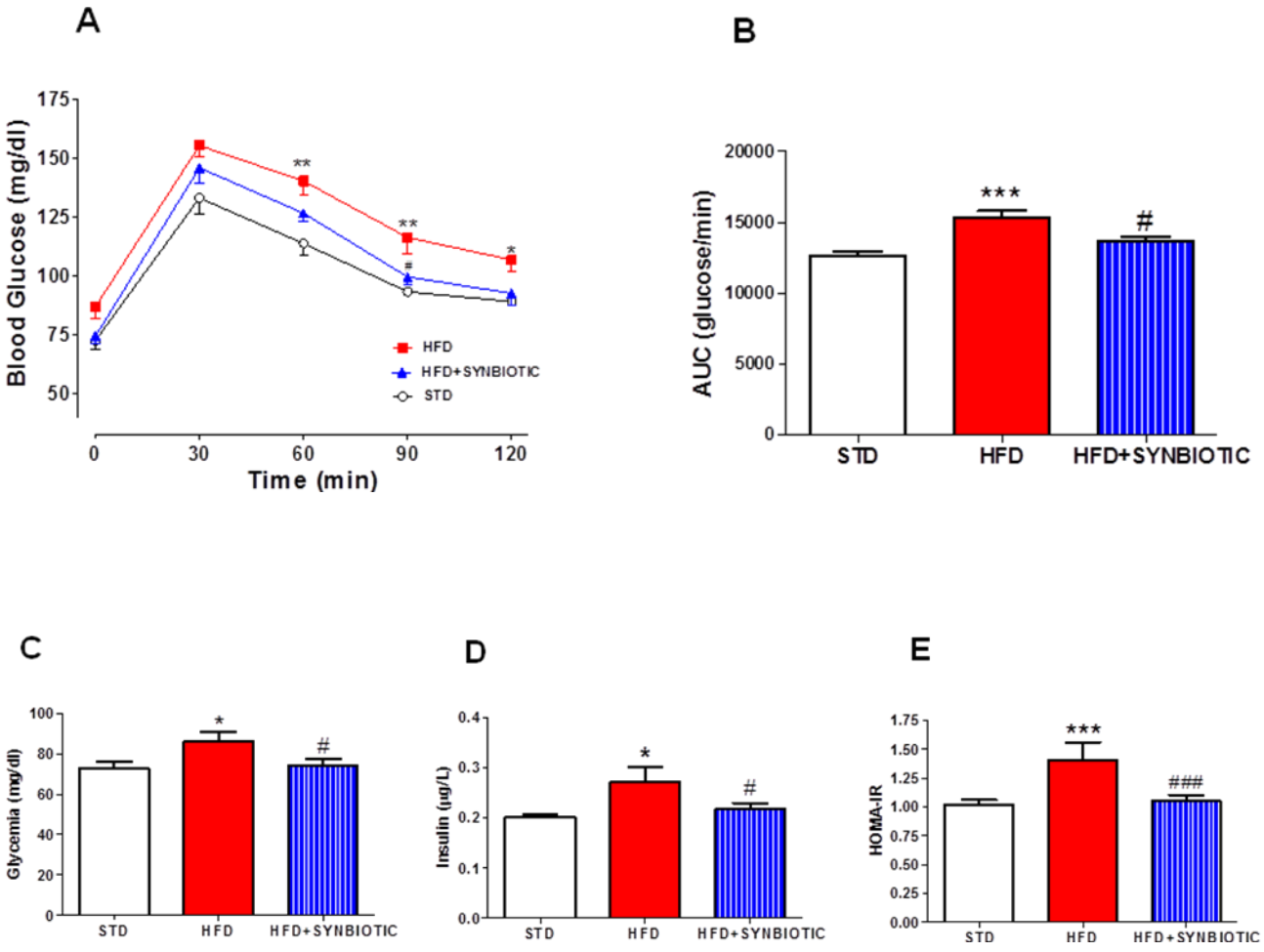


Fig. 5.2. The synbiotic effects on glucose homeostasis. Glucose tolerance test (A) in STD and HFD-fed rats (n=6, each group) was performed and AUC evaluated (B). Fasting glucose (C), insulin levels (D), and HOMA-IR (E) were also reported (n=8, each group). (*P<0.05; **P<0.01; ***P<0.001 *vs.* STD; # P<0.05; ### P<0.001 *vs.* HFD).

Effects of the synbiotic on TNF-α and IL-6 gene liver expression

The raise of pro-inflammatory cytokines is one of the early events in many types of liver injury. In particular, TNF-α and IL-6 are two prototypic inflammatory cytokines involved in metabolic impairment, initiating the pathogenesis of hepatic IR. As shown in **Fig. 5.3A and B**, HFD induced a significant increase in hepatic TNF-α and IL-6 mRNAs, and the synbiotic significantly prevented the transcription of both genes. As known, TNF-α and IL-6 are involved in IR due to their ability to impair insulin signaling through the phosphorylation of IRS-1 in Ser ^{307} and up-regulation of SOCS3, respectively. As depicted in **Fig. 5.3C**, Western blot analysis of P-IRS-1Ser ^{307} of immune-precipitated IRS-1 from hepatic tissues showed an increase in serine phosphorylation in HFD group, partially reverted by the synbiotic. Moreover, the increase in SOCS3 in hepatic tissues from HFD rats was significantly inhibited by the synbiotic (**Fig. 5.3D**).

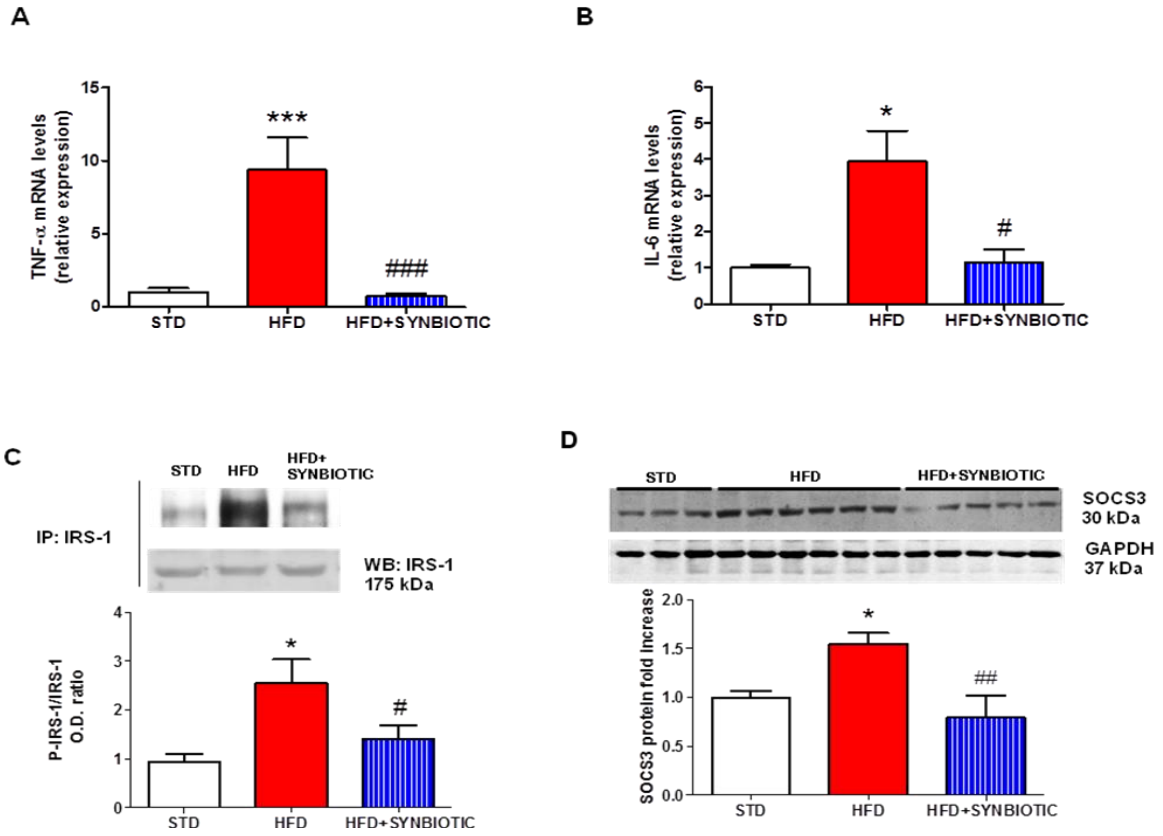


Fig. 5.3. The synbiotic effects on TNF-α and IL-6 gene expression in liver and insulin signaling. TNF-α (A) and IL-6 (B) mRNAs expression (relative expression to STD) are reported (n=8 each group). Panels C and D show representative western blot analysis of P-IRS-1Ser 307 of IRS-1 immunoprecipitate from liver tissues and SOCS3 expression, respectively. (*P<0.05; ***P<0.001 *vs.* STD;# P<0.05; ##P<0.01; P<0.001 *vs.* HFD).

Modulation of hepatic inflammatory transcription factors by the synbiotic

The activation of NF-κB was evaluated through the measurement of cytosolic amount of IκBα and nuclear content of p50 NF-κB. In our model, nuclear p50 NF-κB resulted increased in HFD group, related to a decrease of the inhibitory protein IκBα, the synbiotic significantly prevented both effects (**Fig. 5.4A and B**). Accordingly, with the metabolic and inflammatory alterations, PPAR-α expression resulted significantly reduced by HFD and partially restored by the synbiotic (**Fig. 5.4C**). The evaluation of fibroblast growth factor (FGF)21 transcription, as a downstream target gene of PPAR-α, revealed a similar profile of expression of its transcription factor (**Fig. 5.4D**). These findings were consistent with carnitine palmitoyltransferase (CPT) 1 expression level, whose transcription was up-regulated by synbiotic, suggesting an increase in the oxidation of fatty acids (**Fig. 5.4E**).

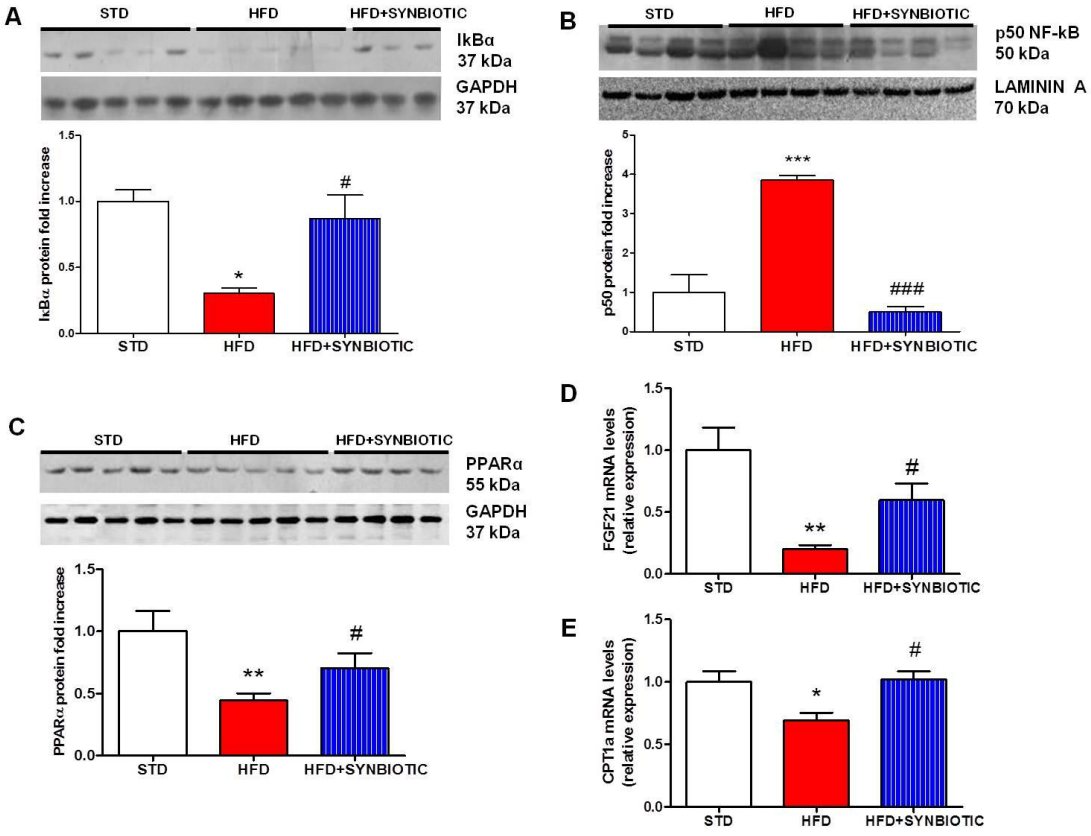


Fig. 5.4. The synbiotic effect on hepatic activation of NF-κB, PPAR-α and FGF21 expression. Immunoblot of cytosolic inhibitory protein IκB-α (A), nuclear p50 NF-κB (B) and PPAR-α (C) protein expression are shown (n=8 each group). FGF21 (D) and CPT1 (E) mRNAs expression (relative expression to STD) are also reported. (*P<0.05; **P<0.01; ***P<0.001 *vs.* STD; # P<0.05; ### P<0.001 *vs.* HFD).

Effect of the synbiotic on hepatic Toll-like receptors pattern

The activation of TLRs family, especially TLR4, by inflammatory cytokines or increased NEFA could modulate insulin sensitivity [115]. As shown in **Fig. 5.5A and B**, HFD induced an increase in liver TLR4 mRNA and protein expression. Interestingly, a similar expression profile was also observed for TLR4 co-receptor CD14 (**Fig. 5.5C**). The synbiotic significantly inhibited these effects. Notably, TLR2 and TLR9, which are able to detect lipoproteins and unmetilated CpG-containing DNA, respectively, were also up-regulated by HFD and both TLRs were reduced by synbiotic (**Fig. 5.5D and E**).

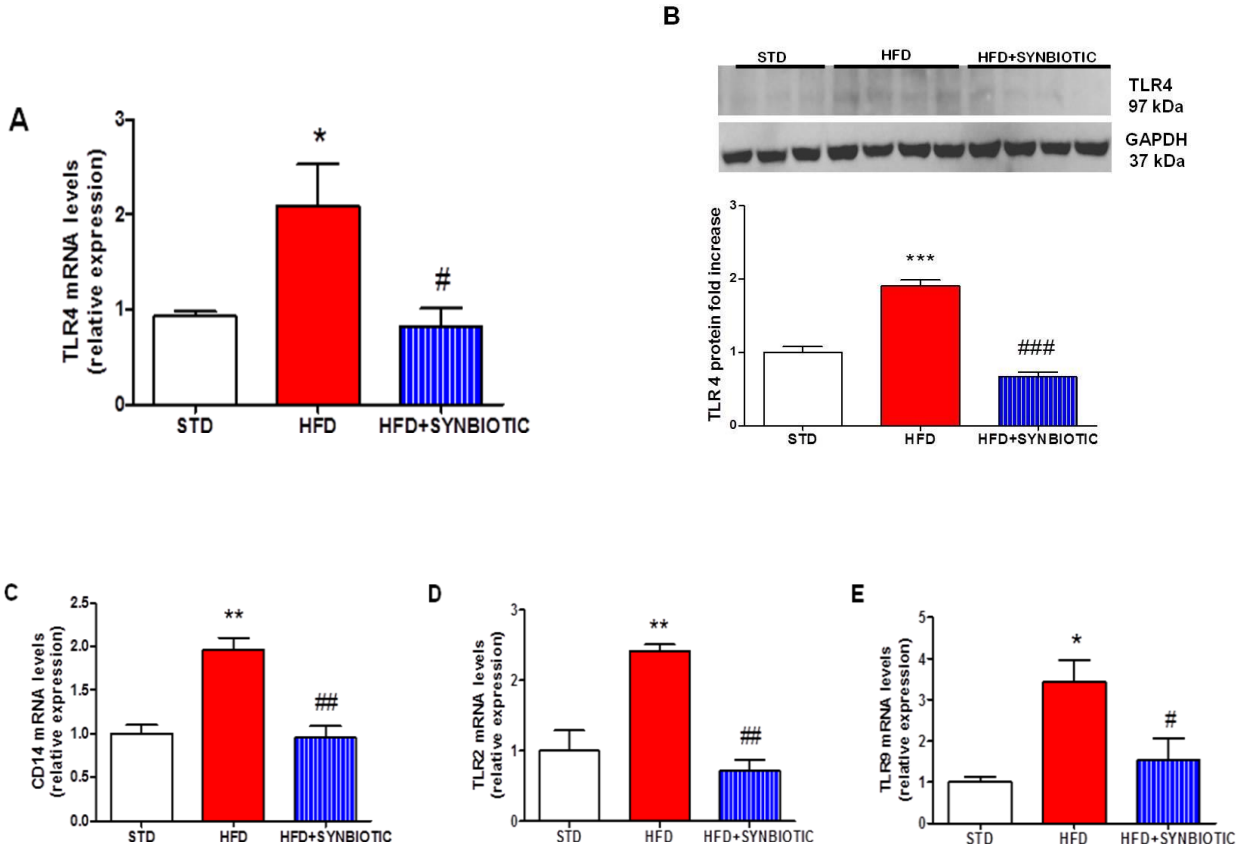


Fig. 5.5. Effect of synbiotic on hepatic Toll-like receptor pattern. Panels A and B are the results from the PCR and Western blot for TLR4 in livers from 8 rats on STD or HFD or HFD+SYNBIOTIC. Panel C shows mRNA expression of coreceptor CD14. mRNA expression of TLR2 (D) and TLR9 (E) are also shown. All mRNA levels are expressed as relative expression to STD. (*P<0.05; **P<0.01; ***P<0.001 *vs.* STD; # P<0.05; ##P<0.01; ### P<0.001 vs. HFD).

Modulation of PPAR-γ, GLUT4 and adiponectin expression in adipose tissue elicited by the synbiotic

To address whether the change in glucose metabolism was related to a modulation of genes expression involved in glucose and fat metabolism in metabolically active tissues, we evaluated the expression of PPAR-γ and GLUT4 in visceral white adipose tissue. In animals receiving HFD, a significant reduction of PPAR-γ and GLUT4 was observed after 6 weeks. The synbiotic limited these effects preventing PPAR-γ decrease (**Fig. 5.6A**) and partially limiting the effect of HFD on GLUT4 expression (**Fig. 5.6B**). Moreover, the reduction of adiponectin mRNA in mesenteric adipose tissue from HFD group was abolished by synbiotic (**Fig. 5.6C**).

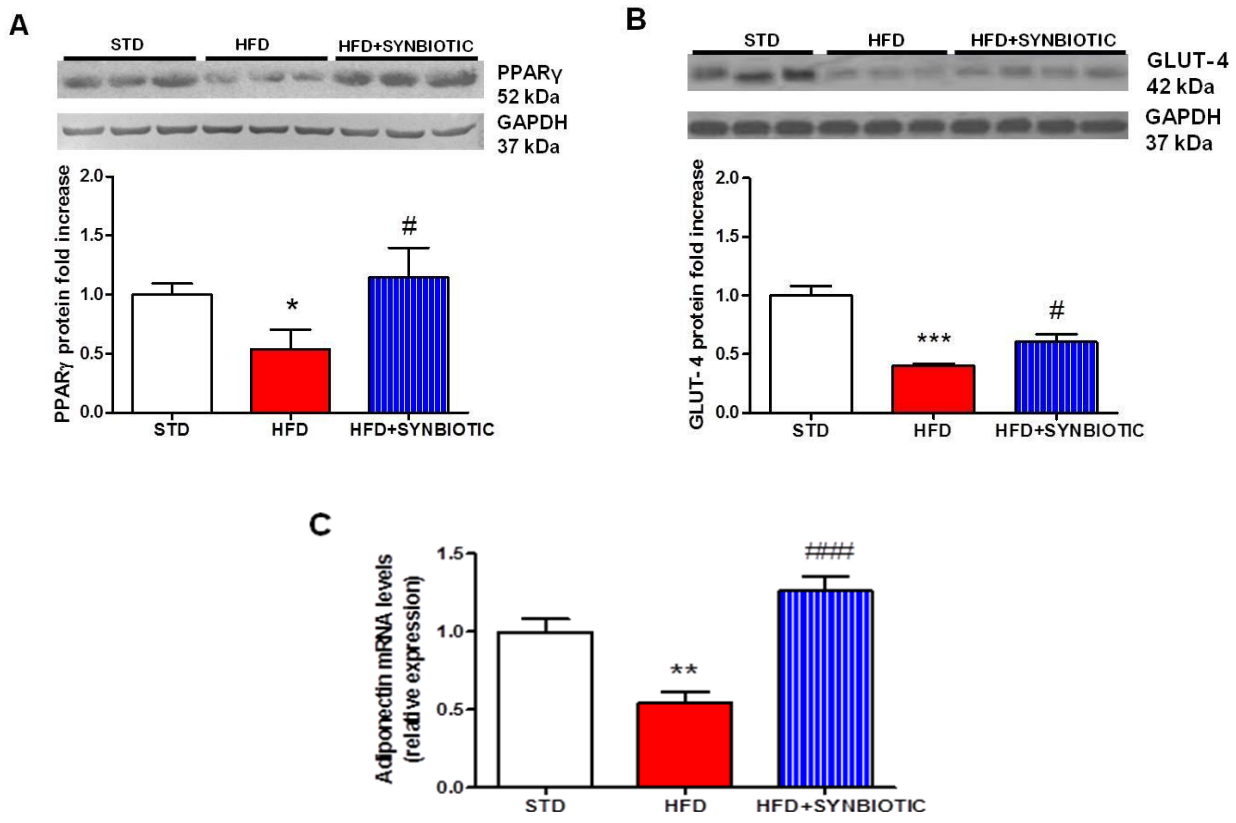


Fig. 5.6. The synbiotic modulation of metabolic and inflammatory proteins in adipose tissue. Representative Western blot of PPAR-γ (A) and GLUT4 (B) are shown (n=8 eachgroup). Panel C shows PCR results from adiponectin mRNA expression (relative expression to STD) in adipose tissue (n=8 each group). . (*P<0.05; **P<0.01; ***P<0.001 *vs.* STD; # P<0.05; ### P<0.001 *vs.* HFD).

Effect of the synbiotic on intestinal permeability and tight junction-associated proteins in gut mucosa

As a consequence of HFD feeding, epithelial barrier integrity was altered. There was a significant increase in gut permeability measured in vivo by appearance in plasma of FITC-labeled dextran (**Fig. 5.7A**), by a mechanism associated with a reduced expression of the epithelial tight junction proteins ZO-1 and occludin (**Fig. 5.7B-E**). These effects were prevented by the synbiotic (**Fig. 5.7A-E**).

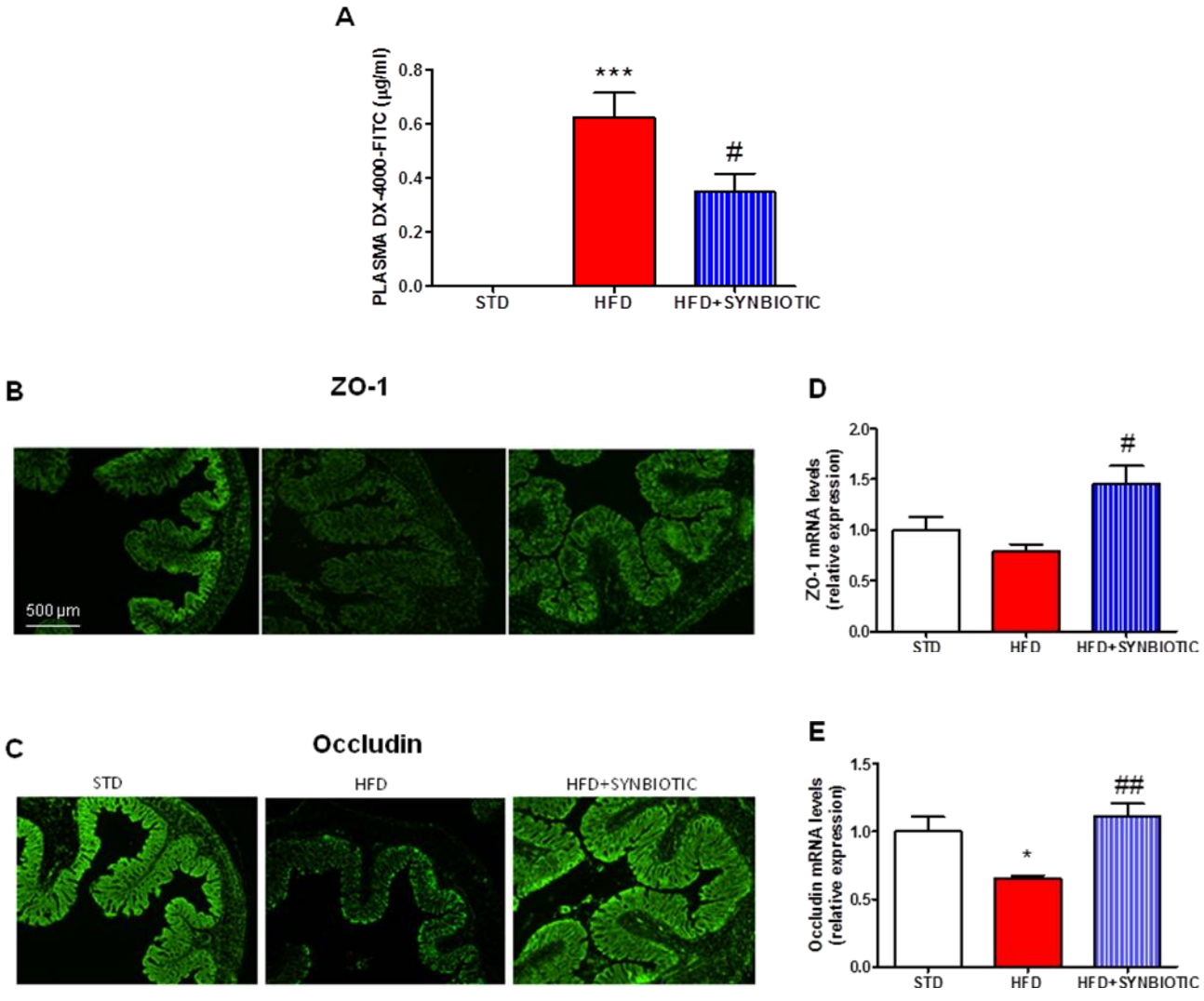


Fig. 5.7. Effect of the synbiotic on intestinal permeability and tight junction-associated proteins. Panel A shows the measurement of gut permeability by appearance of FITC-labeled dextran in plasma of STD, HFD and HFD+SYNBIOTIC rats (n=8 each group). Immunofluorescent images (5×objective) and mRNA expression for ZO-1 (B, D) and occludin (C, E) in the colon tissue are shown (n=4 each group). (*P<0.05; ***P<0.001 *vs.* STD; # P<0.05; ## P<0.01 *vs.* HFD). Immunofluorescence staining are representative of 3 slides for each group.

Modulation of Gram-negative bacteria and TLR4 in colonic mucosa

HFD strongly increased 16S rRNA levels of Enterobacteriales order and related species (Escherichia coli spp) at colonic level, while the synbiotic significantly reduced Gram-negative bacteria (**Fig. 5.8A-B**). The modulation of Gram-negative bacteria was associated with a significant increase in TLR4 and MyD88 in HFD rat intestinal mucosa. Also this effect was significantly blunted by the synbiotic (**Fig. 5.8C and D**).

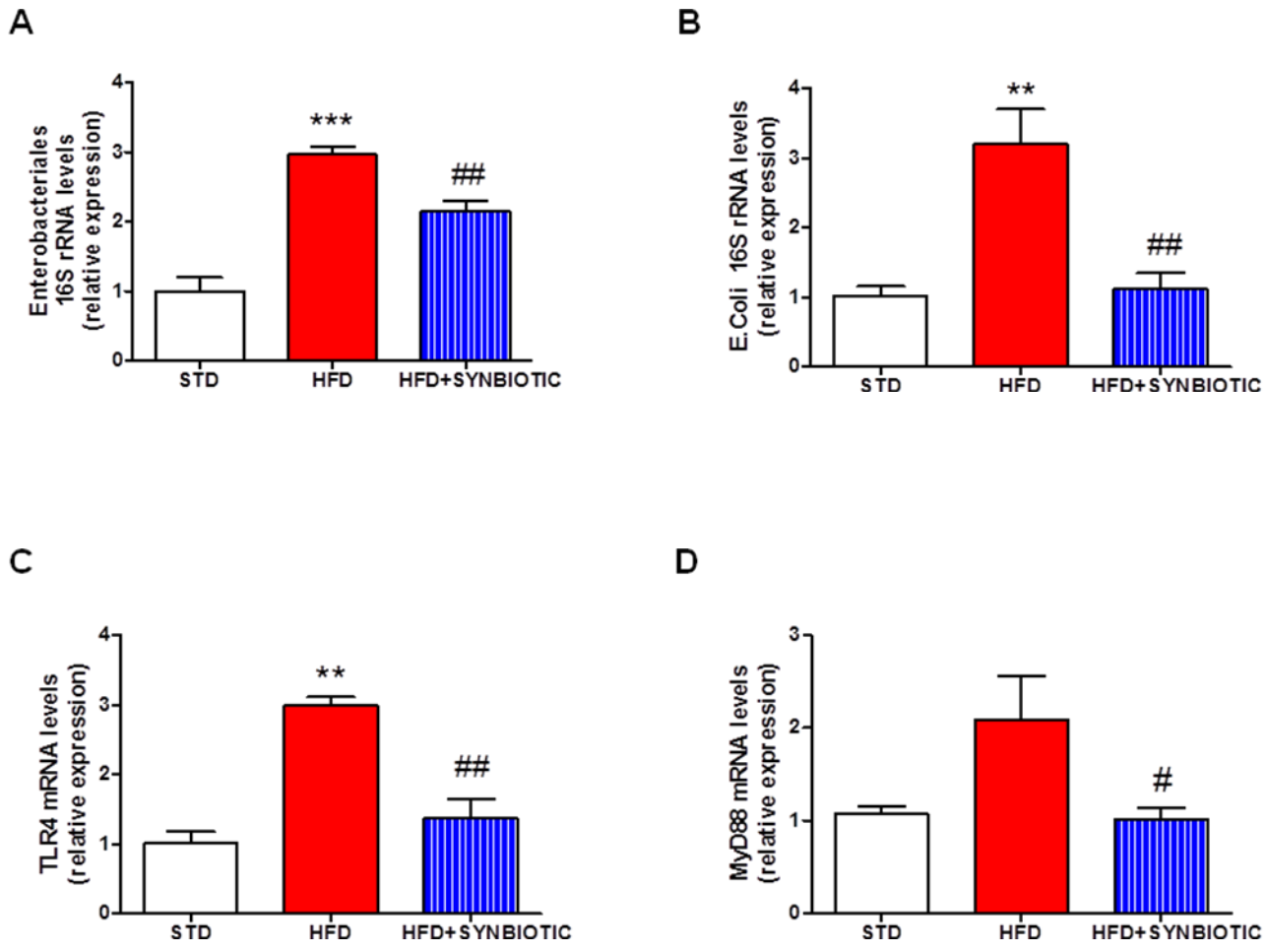


Fig. 5.8. Enterobacteriales and Escherichia coli modification and TLR4 and MyD88 transcription in colonic mucosa. Relative amount of Enterobacteriales order (A) and semi-quantitative analysis of Escherichia coli species (B) are shown. mRNA abundance of TLR4 (C) and MyD88 (D) are also shown. (**P<0.01; ***P<0.001 *vs.* STD; # P<0.05; ## P<0.01 *vs.* HFD).

5.4 Discussion

We show that the synbiotic containing *L. paracasei B21060* plus arabinogalactan and fructo-oligosaccharide, is able to prevent liver damage and inflammation, steatosis, IR, and imbalance of TLRs pattern in the early stage of NAFLD. The synbiotic not only prevents the increase of hepatic markers of steatosis and NEFA, but also preserves glucose tolerance, reduces fasting glucose and insulinemia modulating HOMA-IR and adiponectin levels. It is now clear that TNF-α and IL-6 represent crucial effectors of IR, that link liver inflammatory process to hormonal and metabolic alterations [93,506]. In our experimental model, the synbiotic reduces TNF-α levels in parallel with a lower Ser^{307}-phosphorylation of IRS-1, demonstrating the recovery of insulin signaling transduction. IL-6, activating the JAKSTAT pathway, stimulates SOCS1 and SOCS3 transcripts that in turn led to ubiquitin-induced degradation of IRS-1 [93]. Here, we show that the synbiotic reduces the transcription of both cytokines, TNF-α and IL-6, and inhibits markedly their above reported pathways, limiting inflammation and IR. In our experimental conditions, it is plausible to argue that the synbiotic, reducing NEFA and cytokines (i.e., TNF-α, IL-6 and IL-1β), modulates the activation of NF-κB pathway induced by HFD [507], reducing IκBα degradation and inhibiting p50. Moreover, the increased expression of TNF-α by HFD is associated with the reduction of PPAR-α expression in liver and adiponectin synthesis in mesenteric adipose tissue. Adiponectin, an insulin-sensitizing anti-inflammatory adipokine, limits fat accumulation in the liver by a number of mechanisms including induction of PPAR-α expression [508], reduces liver TNF-α expression [509], and inhibits expression of several cytokines in hepatic stellate cells, with a concomitant increase in the release of the regulatory cytokines IL-10 and IL-1RA [510]. The molecular mechanisms by which the synbiotic exerts its beneficial effects on NAFLD are linked to the marked increase of adiponectin and to the partial recovery of PPAR-α. The level of PPAR-α which regulates fatty acid β-oxidation and catabolism, was restored by the synbiotic. We previously demonstrated that HFD feeding is associated with the reduction of PPAR-α expression in liver [70] and according to our findings, the administration of a PPAR agonist or probiotics restores PPAR-α and improves hepatic steatosis [70,71]. Recently, it has been demonstrated that FGF21, a cytokine/hormone predominantly produced by the liver, was regulated by PPAR-α [511]. FGF21 regulates glucose and lipid metabolism through pleiotropic actions in pancreas and adipose tissue [512]. In particular, FGF21 is required for the normal activation of hepatic lipid oxidation and triglyceride clearance [513]. In our model FGF21 was significantly reduced by HFD and its decreased expression was partially prevented by synbiotic. Accordingly, the synbiotic not only

increased PPAR-α and FGF21, but also normalized CPT1 transcription, suggesting a role for this pathway in synbiotic-induced decrease in fatty acid accumulation in the liver. As known, adiponectin up-regulation by PPAR-γ, provides a connection between the two PPAR isotypes [514]. PPAR-γ promotes fatty acid uptake and increases insulin sensitivity by up-regulating GLUT4, an insulin dependent glucose transporter in adipose tissue and striated muscle [515] and attenuating the induction of SOCS3 [516]. Consistently with the modulation of adiponectin synthesis, the synbiotic also modulates PPAR-γ and GLUT4 expression in visceral adipose tissue. To address the synbiotic mechanisms on HFD-induced hepatic alterations, we evaluated gut permeability. The synbiotic is able to significantly inhibit the modification of gut permeability induced by HFD. In fact it significantly reduces the amount of FITC-dextran at plasmatic level and restores the HFD-induced alteration in tight junction proteins expression and distribution. The synbiotic also prevents the increased transcription of TLR4 in the colonic mucosa of HFD animals, suggesting a reduction of TLR4 inflammatory pathways. TLRs are involved in bacterial sensing and are crucial for "liver tolerance" in the healthy liver [67]. Here, we demonstrate that the synbiotic is able to limit the increased transcription and expression of TLRs and co-receptor CD14 or MyD88 at intestinal and liver level and restores the imbalance of Gram negative bacteria (Enterobcteriales and in particular *E. coli*) induced by HFD. Recently, it has been demonstrated that during HFD-induced diabetes, commensal intestinal bacteria translocate in pathological manner from intestine towards the tissues where they trigger a local inflammation. This metabolic bacteremia was reversed by a Bifidobacterium animalis strain, which reduced the mucosal adherence and bacterial translocation of gram-negative bacteria from the Enterobacteriaceae group [517]. Moreover, an increase in Enterobacteriaceae family within Enterobacteriales order has been associated with gut inflammation; induction of experimental colitis in rodents was followed by an increase in this family, suggesting that it may be a consequence of gut inflammation rather than a cause [518]. In our model the increased amount of Enterobacteriales induced by HFD, and probably associated to gut inflammation, was restored by this *L. paracasei strain,* which is well known modulator of the inflammatory process [240]. In this study, the increased expression of hepatic TLRs due to HFD confirms a greater exposure of the liver to ligands for these receptors (i.e. PAMPs and DAMPs) deriving from the intestine. Our data are in agreement with previous studies showing that the administration of probiotics (i.e. Lactobacillus and Bifidobacterium) or prebiotics (i.e., inulin and oligofructose) can modulate the microbiota and improve gut permeability, thus controlling the occurrence of endotoxemia [519,520]. Also NEFA and other non-bacterial substances, may act as ligands for TLR2, TLR4, and TLR9 [67,521]. In NAFLD patients, elevated NEFA levels are

commonly observed [522]. Very recently, it was demonstrated that free fatty acids could stimulate NF-κB activation in hepatocytes in the early stage of HFD-induced NAFLD through the TLR4 [523].

Here, we demonstrate that the synbiotic reduces inflammation and its mediators, not only through an effect on NEFA and intestinal permeability, but also inhibiting NF-κB activation through the downregulation of TLR pattern. Accordingly, we evidenced the same profile of activity of the synbiotic on TLR2 expression. Both TLR2 and TLR4 recognizes NEFAs [524], and share the same signaling cascade leading to NF-κB activation. The reduction of TLR2 by the synbiotic may contribute to the inhibition of the effects of HFD, impacting on IR and tissue damage. Consistently with our data, Ehses et al. [525] have reported that TLR2 deficient mice are protected from IR and β cell dysfunction induced by HFD, linking TLR2 to the increased dietary lipid and the alteration of glucose homeostasis. Finally, the synbiotic significantly inhibits the HFD-related increase in TLR9 synthesis. Intracellular TLR9 activates innate immune defenses against viral and bacterial infection and plays a role in the pathogenesis of NASH [118].

5.5 Conclusions

In conclusion, our results support probiotics as innovative, preventive and therapeutic strategy for NAFLD, using synbiotic preparations containing selected strain with clear and demonstrated beneficial immunomodulatory effects. Among probiotics, *L. paracasei B21060*, can be considered a potential approach, limiting the main pathogenetic events involved in the onset of IR and steatosis induced by HFD. This synbiotic, alone or in combination with other therapies, could be useful in the treatment of fatty liver in children who are hardly able to follow a program of hypocaloric diet and regular physical activity. The major results of our study are summarized in **figure 5.9**.

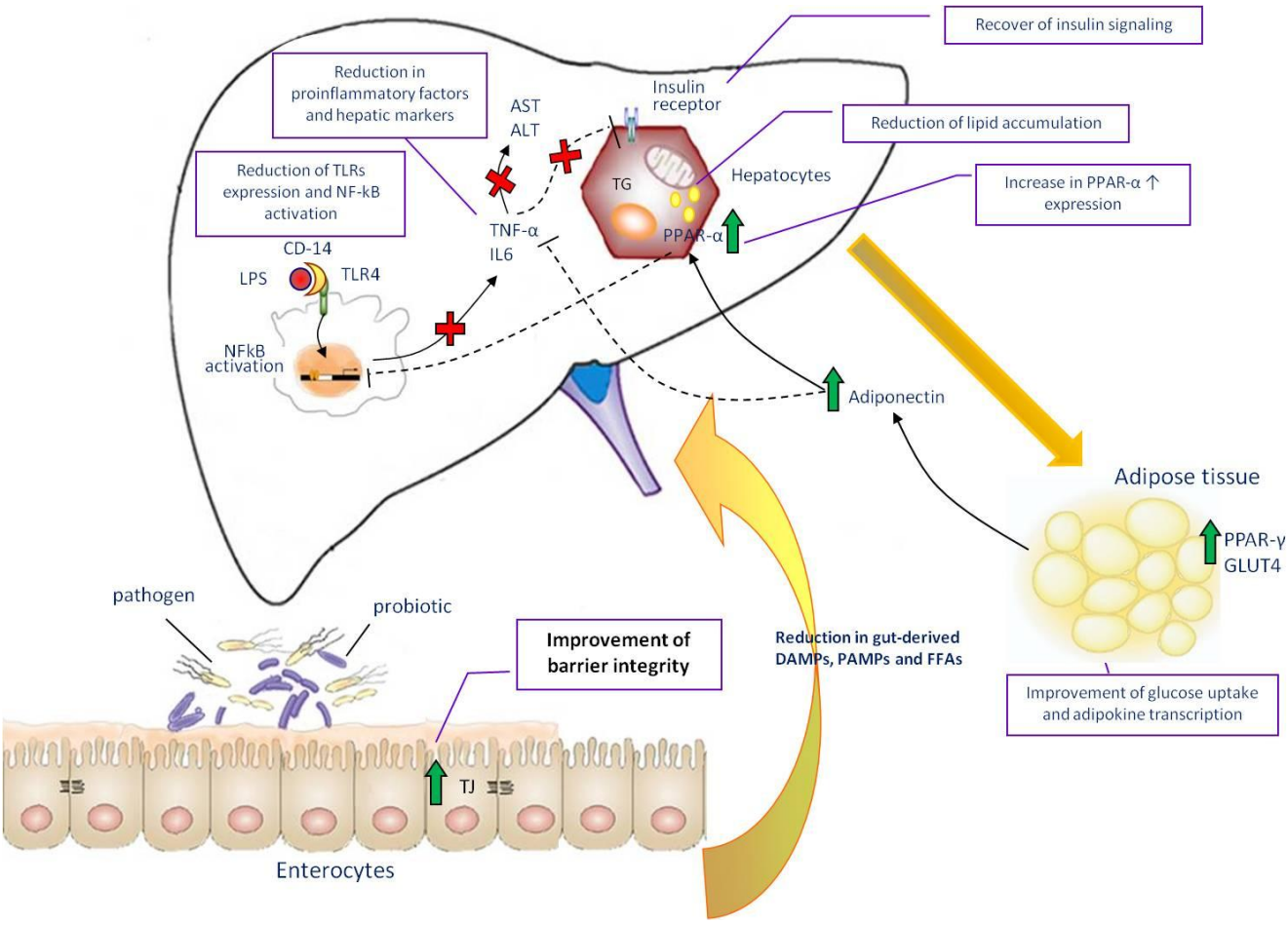


Fig. 5.9. Effects of a Synbiotic based *Lactobacillus paracasei B21060* in the liver. The enhancement of epithelial barrier integrity by the synbiotic through an increase in tight junction proteins expression (ZO-1 and occludin) , determines a reduction of DAMPs, PAMPs, and FFA afflux to liver. This synbiotic effect is related to a reduction in the expression of hepatic TLR4, its accessory molecule CD14, TLR2 and TLR9 and NF-κB-activation with a decrease in inflammatory gene transcription. The reduced expression of inflammatory cytokines (TNF- α and IL-6) leads to an improvement of insulin signaling (reduction of IRS-1 phosphorylation in Ser 307 and down-regulation of SOC3), accompanied by an increase in insulin sensitivity. The synbiotic increases PPAR-α expression in liver, contributing to the reduction of inflammation and the increase in fatty acid catabolism. Consistently, in adipose tissue the synbiotic increases PPAR-γ expression and adiponectin transcription, contributing to reduce inflammation. Finally, the therapeutic/preventive role of the synbiotic in hepatic steatosis could be related to its ability to limit inflammatory liver damage, insulin signaling impairment, and imbalance of TLR patterns induced by high fat diet.

ALT, alanine transaminase; AST, aspartate aminotransferase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; TLR, toll-like receptor; IL, interleukin; TNF, tumor necrosis factor; TG, triglycerides; PPAR, peroxisome proliferator-activated receptor; FFAs, free fatty acids; DAMPs, damage associated molecular patterns; PAMPs, pathogen associated molecular patterns;TJ, tight junction; GLUT4, glucose transporter 4; suppressor of cytokine signaling, SOCS; IRS, insulin receptor substrate.

CHAPTER 6: EFFECTS OF SODIUM BUTYRATE AND ITS SYNTHETIC AMIDE DERIVATIVE ON LIVER INFLAMMATION AND GLUCOSE TOLERANCE IN AN ANIMAL MODEL OF STEATOSIS INDUCED BY HIGH FAT DIET

6.1 Introduction

A close association between NAFLD and several findings indicative of IR and metabolic disorders has long been reported. The liver produces and is exposed to various types of lipids, such as fatty acids, cholesterol and triglycerides via the portal vein from the diet and visceral adipose tissues. The liver and adipose tissue jointly participate in maintaining glucose and lipid homeostasis through the secretion of several humoral factors and/or neural networks. Perturbation in the inter-tissue communications may be involved in the development of IR and diabetes. An excessive free fatty acids (FFAs) flux into the liver via the portal vein may cause fatty liver disease and hepatic IR. However, the initial events triggering the development of IR and its causal relations with dysregulation of glucose and fatty acids metabolism remain unclear. It has been suggested that the blood glucose- and lipid lowering effects of soluble dietary fibres may be related in part to SCFAs generated during anaerobic microbial fermentation [526,527]. Among SCFAs, butyrate constitutes one of the major products derived from intestinal fermentation of undigested dietary carbohydrates, specifically resistant starches and dietary fibres, but also in a minor part by dietary and endogenous proteins. After butyrate uptake by the colon, it is metabolized in part by the colonocytes, and the remaining fraction reaches the liver via the portal vein. The colonocytes absorb butyrate through different mechanisms of apical membrane uptake, including non-ionic diffusion, SCFA/$HCO3^-$ exchange, and active transport by MCT1 [428]. In particular, butyrate is able to exert a powerful pro-absorptive stimulus on intestinal NaCl transport and an anti-secretory effect towards Cl^- secretion [400,403]. The effects exerted by butyrate are multiple and involve several distinct mechanisms of action. Its well-known epigenetic mechanism, is the hyperacetylation of histones by inhibiting class I and class II histone deacetylases (HDAC), that results in the regulation of gene expression and in the control of cell fate [413,528]. HDAC regulates gene transcription through modification of chromatin structure by acetylation of proteins, including not only histone proteins,

but also transcription factors (i.e. NF-κB, p53 and NFAT) [529]. Butyrate also acts as signal molecules, targeting their G protein-coupled receptors Free Fatty Acid Receptor 2 (FFAR2, GPR43) and FFAR3 (GPR41) [378]. At intestinal level, butyrate exerts multiple effects, such as the prevention and inhibition of colonic carcinogenesis, the improvement of inflammation, oxidative status, epithelial defense barrier, and the modulation of visceral sensitivity and intestinal motility. At the extraintestinal level, potential fields of application for butyrate seem to be the treatment of different pathologies, including metabolic diseases, such as hypercholesterolemia, obesity, IR, and ischemic stroke [530]. Recently, it has been demonstrated that dietary supplementation of butyrate can prevent or treat diet-induced IR in mice [477]. The mechanism of butyrate action was related to promotion of energy expenditure and induction of mitochondrial function through stimulation of PGC-1α. Moreover, activation of AMPK and inhibition of HDAC could contribute to PGC-1α regulation by butyrate. More recently Li et al., [531] demonstrated that FGF 21, which plays an important role in lipid metabolism [532], is induced by butrate and involved in the stimulation of fatty acid β-oxidation in liver. Some butyrate-based products are marketed but their spread is still very limited and greatly understaffed in view of the wide spectrum of possible indications especially in chronic diseases where it is possible to predict a lasting use of these compounds. The unpleasant taste and odour make extremely difficult the oral administration of butyrate, these difficulties are even more remarkable in children where the administration is complicated. Thus, new formulations of butyrate with a better palatability, which can be easily administered orally, are needed. The purpose of this study is to investigate the efficacy of sodium butyrate (butyrate) and of its more palatable derivative, the N-(1-carbamoyl-2-phenyl-ethyl) butiramide (FBA), in a rat model of NAFLD induced by high fat diet. We hypothesized that orally administered butyrate compounds, could attenuate steatosis and liver injury, with reduction of inflammatory responses via suppression of Toll-like receptors through downregulation of NF-κB activation.

6.2 Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the Institutional Guidelines and complied with the Italian D.L. no. 116 of January 27, 1992 of Ministero della Salute and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC). All animal procedures reported herein were approved by the Institutional Committee on the Ethics of Animal Experiments (CSV) of the University of Naples "Federico II" and by the Ministero della Salute under protocol no. 2008-0099793. Prior to sample collection, animals were euthanized by an intraperitoneal injection of a cocktail of ketamine/xylazine, followed by cervical dislocation to minimize pain. All efforts were made to minimize animal suffering.

Drugs and reagents

Standard and high-fat diet (HFD) were purchased from Laboratorio Dottori Piccioni (Gessate, Milan, Italy). Standard diet had 15% fat, 22% proteins, and 63% carbohydrates, while HFD had 58% of energy derived from fat, 18% from protein, and 24% from carbohydrates. The composition of high fat diet has been previously described [255]. Standard and high fat diets contained 4.06 kcal/g and 5.56 kcal/g, respectively. Sodium butyrate was purchased by Sigma-Aldrich (Milan Italy) and phenylalanine-butyramide (FBA; Italian patent RM2008A000214; April 21, 2008) was provided by Prof. Calignano. Their chemical structure are reported in **figure 6.0**. FBA is present in a solid, poorly hygroscopic, easily weighable form, stable to acids and alkalis and capable of releasing butyric acid at small and large bowel level in a constant manner over time. This product has demonstrated a toxicological profile comparable to that of butyrate; it shows physicochemical characteristics distinctly more suitable for extensive clinical use than those of butyrate. A particular aspect of FBA is that it does not present the unpleasant odour of butyrate and is practically tasteless, thus making possible to overcome the main limitation to the use of butyrate in the therapeutic field, namely its very poor palatability. Moreover, the solubility of FBA in water is satisfactory in that it produces clear solutions up to the concentration of 0.1 M and suspensions for higher concentrations.

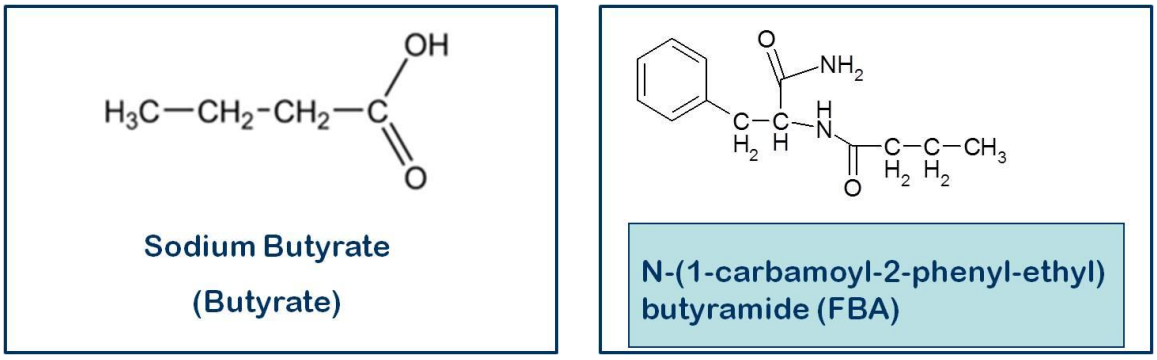


Figure 6.0. Chemical structure of sodium butyrate and its derivative FBA

Synthesis and characterization of butyric acid derivative FBA

Briefly, 0.01M of phenylalanine carboximide and 0.01M butyroyl chloride were dissolved in 50 ml of chloroform and the resulting mixture were left to react at room temperature for 24h. The mixture, evaporated in vacuo, yields a solid white-coloured residue, that was washed with a 1% sodium bicarbonate solution. The aqueous bicarbonate solution was extracted twice with an equal volume of ethyl acetate to recover an additional fraction of the mixture of derivatives. To isolate the single components, the mixture was treated and processed chromatographically on a silica gel column, using dichloromethane as eluent. The compound was re-crystallised with a mixture of chloroform/n-hexane 1:1 v: v, obtaining a final yield equal to or greater than 50%.

Animals and treatments

After weaning, young male Sprague-Dawley rats (average body weight 113.0±2.2 g), purchased from Harlan, Italy, were randomly divided into four groups (at least 6 animals for each group) as follows: 1) a control group receiving STD and vehicle *per os* by gavage; 2) HFD-fed group receiving vehicle; 3) HFD fed group treated by gavage with sodium butyrate (HFD +butyrate, 20 mg/kg/die) or 4) with N-(1-carbamoyl-2-phenylethyl) butyramide (HFD+FBA 42.5 mg/Kg/die, the equimolecular dose of butyrate). The treatments started together with the HFD and continued for 6 weeks. We used a nutritional model of IR in non-genetically modified animals [256] that after 6 weeks, induced the early events of NAFLD due to fat overnutrition in young animals, excluding age and gender influences. Blood sample was collected by cardiac puncture and serum obtained. Liver and white adipose tissue were excised and immediately frozen.

Blood biochemistry

Alanine amino transferase (ALT), aspartate amino transferase (AST), total cholesterol, low density lipoprotein (LDL) and triglycerides (TGL) were measured by standard automated procedures, according to manufacturer's protocols (Dade Behring Inc., Newark, DE). Fasting insulin concentrations were measured by rat insulin radioimmunoassay kit (Millipore Corporation, Billerica, MA, USA). Blood glucose concentrations were measured using a glucometer (One Touch UltraSmart; Lifescan, Milpitas, CA). Blood NEFA were determined as previously described (Itaya and Ui., [504].

Histological Analysis of Liver Tissue and triglycerides content

Liver sections were stained with hematoxylin and eosin or Oil Red O. Steatosis was graded on a scale of 0 (absence of steatosis), 1 (mild), 2 (moderate) and 3 (extensive). Liver triglycerides were determined as previously described [533].

Oral Glucose Tolerance Test (OGTT) and Insulin Resistance Assessment

Five weeks after the beginning of the experiment, all rats were fasted for 18 h and then underwent a glucose tolerance. Glucose was orally administered (2 g/kg body weight). Blood samples were collected sequentially from the tail vein before, and 30, 60, 90, and 120 min after the glucose load. The area under the glucose concentration versus time curve (AUC) was calculated from time zero, as the integrated and cumulative measure of glycemia up to 120 min for all animals. To compare the course of the glucose concentration among groups, statistical analysis of AUC mean values was performed using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). As index of insulin resistance, HOMA was also calculated, using the formula [HOMA = fasting glucose (mmol/L) × fasting insulin (μU/ml)/22.5].

Western blotting

Liver and white adipose tissues were homogenized and total protein lysates were subjected to SDS-PAGE. Blots were probed with anti-suppressor of cytokine signaling 3 (SOCS3, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or antiprotein tyrosine phosphatase 1B (PTP1B, Santa Cruz Biotechnology), or anti-COX-2 (Cayman Chemical, Ann Arbor, MI), or anti-iNOS (BD Trusduction, Franklin Lakes, NJ, USA), or anti-peroxisome proliferator-activated receptor α (PPAR-α, Santa Cruz Biotechnology), or anti-PPAR-γ (Novus Biologicals, Littleton, CO, USA), or anti-glucose transporter-4 (GLUT-4, Santa Cruz Biotechnology) or anti-glucose transporter-2 (GLUT-2, Millipore Corporation, Billerica, MA, USA). To evaluate NF-κB activation, IκB-α (Santa Cruz Biotechnology) and NF-κB p50 (Santa Cruz Biotechnology) were measured in liver cytosolic or nuclear extracts, respectively. Western blot for glyceraldehyde-3- phosphate dehydrogenase (GAPDH) or β-actin (in cell lysates, Sigma-Aldrich, Milan, Italy) or lamin A (in nuclei lysates, Chemicon. Int, Temecula, CA, USA) was performed to ensure equal sample loading.

Real-time semi-quantitative PCR

Total RNA, isolated from liver and adipose tissue, was extracted using TRIzol Reagent (Invitrogen Biotechnologies), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 μg total RNA. PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories). The primer sequences are reported in **Table 6.2**. The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample contained 1-100 ng cDNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/l of each primer (Eurofins MWG Operon, Huntsville, AL) in a final volume of 25μl. The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data were presented as mean ± SEM. Statistical analysis was performed by ANOVA test followed by Bonferroni's test for multiple comparisons. Statistical significance was set at $P<0.05$.

Table 6.2. Real-Time PCR Primer Sequences

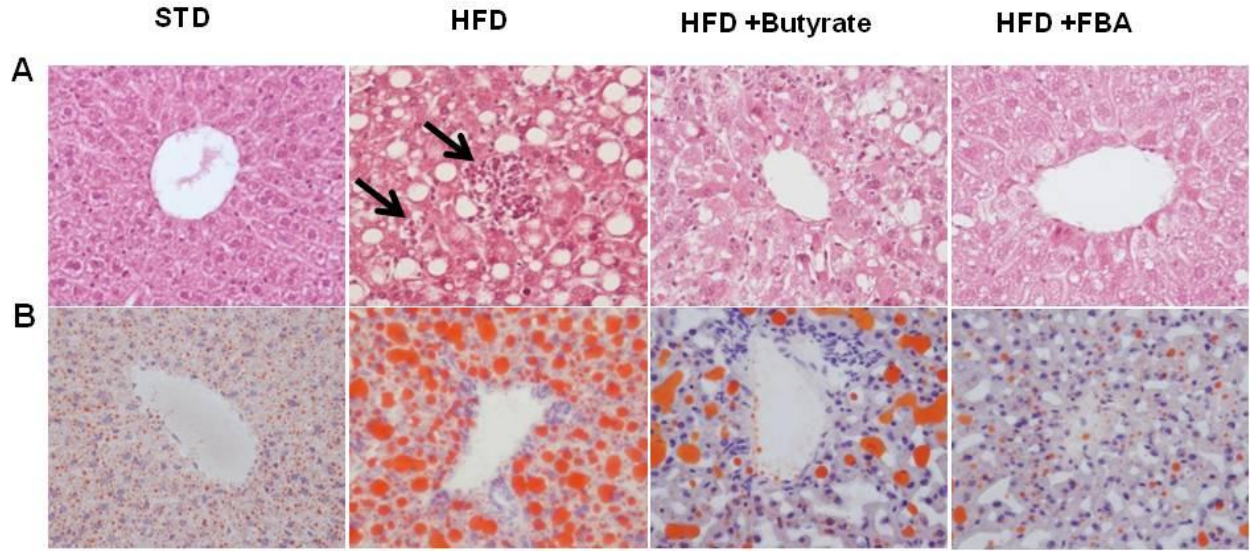
Target gene	Forward primer (5'→3')	Revere primer (3'→5')	Accession Number
CD14	GTGCTCCTGCCCAGTGAAAGAT	GATCTGTCTGACAACCCTGAGT	AF_087943
F4/80	CCCAGCTTATGCCACCTGCA	TCCAGGCCCTGGAACATTGG	NM_001007557.1
FGF21	AGATCAGGGAGGATGGAACA	ATCAAAGTGAGGCGATCCATA	NM_130752.1
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	NM_017008 XM_216453
HMGB-1	TTGTGCAAACTTGCCGGGAGGA	ACTTCTCCTTCAGCTTGGC	NM_012963.2
IL-1β	TCCTCTGTGACTCGTGGGAT	TCAGACAGCACGAGGCATTT	NM_031512
IL-6	ACAAGTGGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC	NM_012589
MCP-1	CCCACTCACCTGCTGCTACT	TCTGGACCCATTCCTTCTTG	NM_031530.1
PGC1-α	AACCAGTACAACAATGAGCCTG	AATGAGGGCAATCCGTCTTCA	NM_031347.1
Pro-collagen type 1	TCGATTCACCTACAGCACGC	GACTGTCTTGCCCCAAGTTCC	NM_053304.1
α- SMA	TGCTGGACTCTGGAGATGG	GATGGTGATCACCTGCCCATC	NM_031004.2
TGF-β	GAAGCCATCCGTGGCCAGAT	TGACGTCAAAAGACAGCCACT	NM_021578.2
TLR2	GTACGCAGTGAGTGGTGCAAGT	TGGCCGCGTCATTGTTCTC	NM_198769 XM_227315
TLR4	CTACCTCGAGTGGGAGGACA	ATGGGTTTTAGGCGCAGAGTT	NM_019178
TLR9	ATGGCCTGGTAGACTGCAACT	TTGGCGATCAAGGAAAGGCT	NM_198131
TNF-α	CATCTTCTCAAAACTCGAGTGACAA	TGGGAGTAGATAAGGTACAGCCC	NM_012675

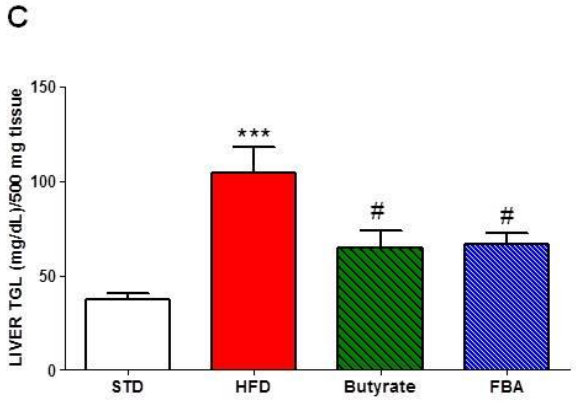
6.3 Results

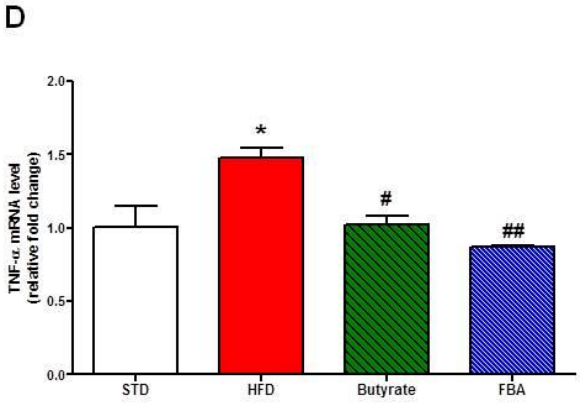
Effects of butyrate and FBA on liver steatosis and serum parameters

Liver sections from rats fed with HFD demonstrated significant hepatic damage in comparison with standard diet (STD) fed animals. As depicted in **Figure 6.1A**, HFD livers showed foci of mixed inflammatory cell infiltration, evidenced by arrows, and hepatocyte necrosis or apoptosis throughout the lobule. Scattered inflammation and occasionally apoptotic nuclei were observed. No alterations were shown in liver of the rats fed with the STD. Furthermore, HFD rats showed grade 3 hepatic steatosis with a histological pattern characterized by microvesicular steatosis. The hepatocytes showed the cytoplasm filled with small vacuoles which were uniform in size and smaller than the centrally located nucleous. Steatosis affected most of the hepatocytes (**Figure 6.1B**). In animals treated with equimolecular doses of butyrate (sodium butyrate or FBA), liver inflammatory damage appears reduced and steatosis was graded as grade 1 with a microvesicular pattern of lipid accumulation distributed in perivenular and periportal region. This effect was also associated with a reduction of triglycerides content which was significantly enhanced by HFD (**Figure 6.1C**). In HFD-fed animals TNF-α, a cytokine involved in the development of IR, was significantly increased, butyrate and in particular FBA reduced hepatic expression of this cytokine (**Figure 6.1D**). Similarly the mRNA concentration of chemokine MCP-1 and a specific marker of mature macrophages F4/80 were increased in HFD rats and normalized by butyrate treatments (**Figure 6.1E and F**). Moreover, HFD induced the expression TGF-β1, an early marker of the fibrotic process (**Figure 6.1G**), which was significantly down-regulated in the butyrate-treated group ($P < 0.01$) and more significantly by FBA ($P < 0.001$). We also evaluated α-SMA and pro-collagen type 1 transcript by real time-PCR but it did not show a significant modification after 6 weeks of HFD. Biochemical serum parameters are reported in **table 6.1**. Circulating levels of AST, ALT and cholesterol resulted increased at 6 weeks of HFD, while LDL and triglycerides showed a trend of increase. All these parameters were reduced by butyrate and FBA. Compared with animals fed with STD diet, HFD rats showed a marked increase in fasting glucose without change in insulin levels. Both butyrate and FBA prevented glucose alteration. Homeostasis model assessment for insulin resistance (HOMA-IR) was 25% and 30% lower in butyrate and FBA groups, respectively. No difference in body weight was shown among all groups,

weight gain of HFD fed animals did not change after 6 weeks neither modified by butyrate and FBA treatments (data not shown).







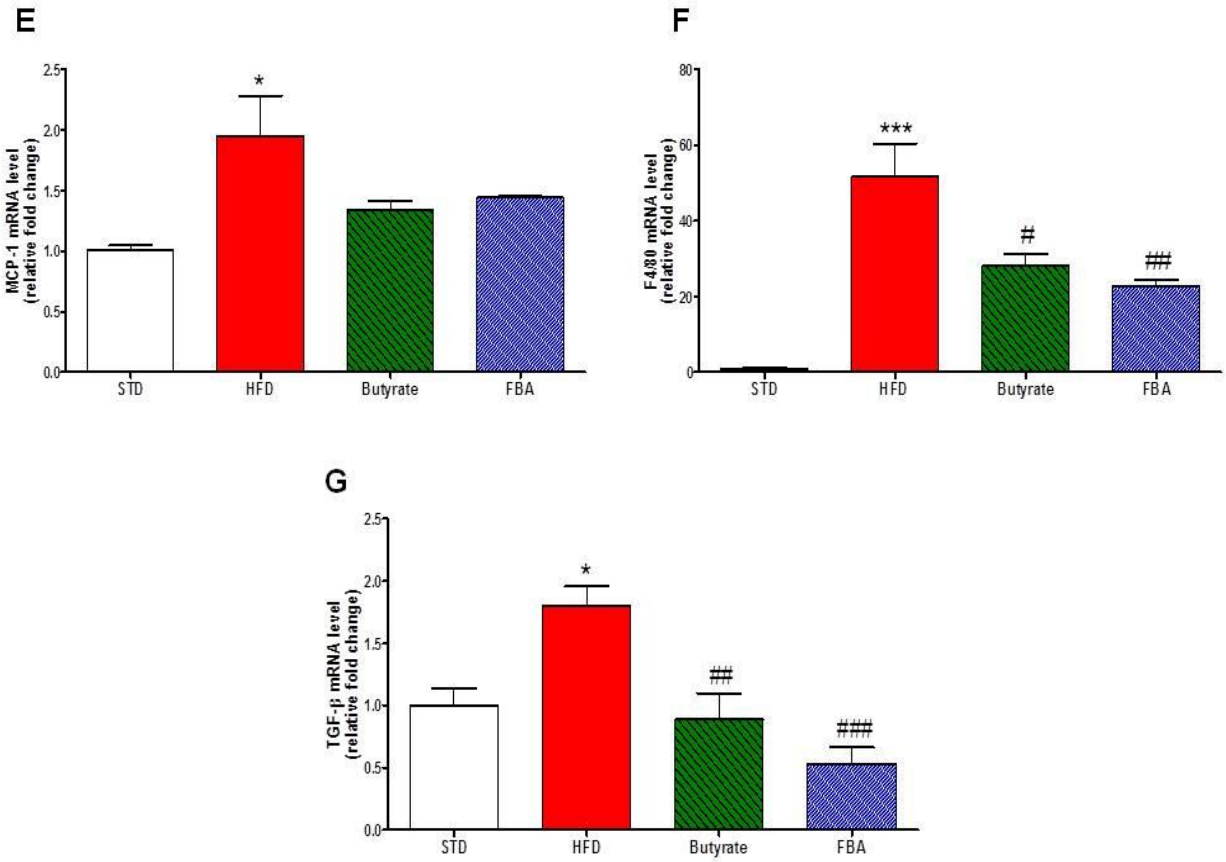


Figure 6.1. Effects of butyrate and FBA on liver steatosis. Paraffin-embedded sections of the liver (n=4 each group) were stained with hematoxylin-eosin (A) or oil red O (B). Micrographs in both panels are representative pictures with original magnification 400X. Foci of inflammatory cells are shown (arrows). (C) Triglycerides were measured in liver and normalized on 500 mg of frozen tissue (n=6 each group). The mRNA levels of pro-inflammatory cytokines TNF-α (D), MCP-1 (E), F4/80 (F), and TGF-β (G) were analyzed by real-time PCR in liver extracts (n=6, each group). (*P<0.05, ***P<0.001 *vs.* STD; # P<0.05, # # P<0.01, # # # P<0.001 *vs.* HFD).

Table 6.1. Changes in serum parameters of rats fed with control standard diet (STD), high fat diet (HFD) or high fat diet and treated with Butyrate or FBA for 6 weeks.

	STD	HFD	HFD+Butyrate	HFD+FBA
ALT (U/l)	26.17±3.40	38.83±3.69 *	22.00±1.59 ##	18.83±1.56 ###
AST (U/l)	157.33±12.23	226.16±24.15 **	152.0±3.65 ##	146.60±12.79 ##
Colesterol (mg/dl)	71.50±1.72	86.14±6.37 *	74.50±1.52 #	74.67±3.03 #
LDL (mg/dl)	24.55±0.74	27.23±2.39	20.40±0.86 #	23.46±0.82
TGL (mg/dl)	38.50±3.52	43.86±3.70	35.17±0.40	39.67±1.62
Fasting glucose (mg/dl)	110.3±3.22	149.0±16.27*	115.2±6.71#	118.4±5.48#
Fasting insulin (µg/l)	0.20±0.06	0.27± 0.02	0.21±0.01	0.20±0.01
HOMA index	0.97±0.03	1.56±0.16**	1.17±0.11#	1.13±0.06#

Values are means ± S.E of six animals. (* P<0.05, **P<0.01 *vs.* STD. #P<0.05; ##P<0.01; ###P<0.001 *vs.* HFD).

Modulation of hepatic inflammatory parameters and NF-κB activation by butyrate and FBA

Hepatic and extra-hepatic IL-1 sources contribute to liver inflammation related to metabolic alterations. In fact, IL-1β is strongly up-regulated by activated macrophages or other liver cell types (including Kupffer cells and hepatic stellate cells), participating in liver injury. Beyond TNF-α, also IL-6 is involved in metabolic impairment, initiating the pathogenesis of hepatic IR. As shown in **Figure 6.2A and B**, HFD induced a significant increase in hepatic IL-1β and IL-6 mRNAs, and both treatments significantly prevented the transcription of these genes. Moreover, the high-fat diet determined an increase in liver proinflammatory enzymes, COX-2 and iNOS (**Figure 6.2C and D**) and butyrate and FBA reduced the expression of these proteins. In agreement both treatments significantly prevented the increase of nuclear content of p50 NF-κB related to a reduction of the inhibitory cytosolic protein IκBα, showing the inhibition of NF-κB activation (**Figure 6.2E and F**).

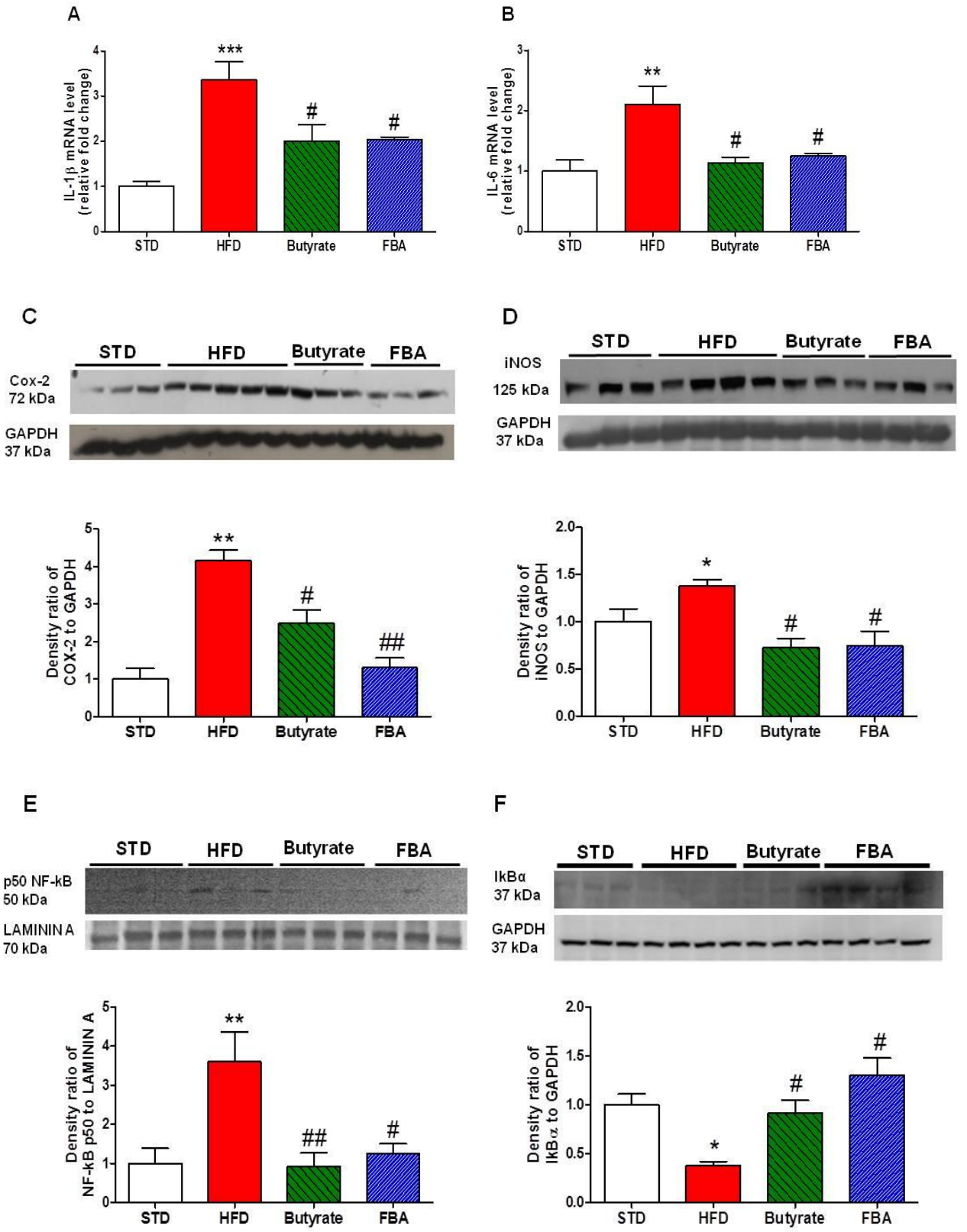


Figure 6.2. Modulation of hepatic inflammatory parameters and NF-κB activation by butyrate and FBA. IL-1β (A) and IL-6 (B) mRNA expression (relative fold change to STD) are reported (n=6, each group). Panels C and D show representative Western blot analysis of pro-inflammatory enzymes COX-2 and iNOS, respectively, in the liver extracts. Nuclear p50 NF-κB (E), and cytosolic inhibitory protein IκB-α (F) expression is reported (n=6 each group). (*P<0.05, **P<0.01, ***P<0.001 *vs.* STD; # P<0.05, # # P<0.01 *vs.* HFD).

Effect of butyrate and FBA on hepatic Toll-like receptors pattern

The activation of TLRs family, especially TLR4, by inflammatory cytokines or increased NEFA could modulate insulin sensitivity [115]. Recently, it has been hypothesized that FFA-related high–mobility group box 1 (HMGB1) release mediates the activation of TLR4 signaling in hepatocytes and plays an essential part in the early stage of NAFLD induced by HFD [523]. Here, HMGB-1 mRNA levels were strongly up-regulated by HFD feeding while both butyrate, and in particular FBA, determined a significant reduction of these levels (**Figure 6.3A**). As shown in **Figure 6.3B and C**, HFD induced the mRNA increase of TLR4 and its co-receptor CD14 in liver. FBA and, to a lesser extent, butyrate inhibited these effects. Notably, TLR2 and TLR9, which are able to detect lipoproteins and unmetilated CpG-containing DNA, respectively, were also upregulated by HFD and reduced by two treatments, with a great effectiveness of the FBA (**Figure 6.3D and E**).

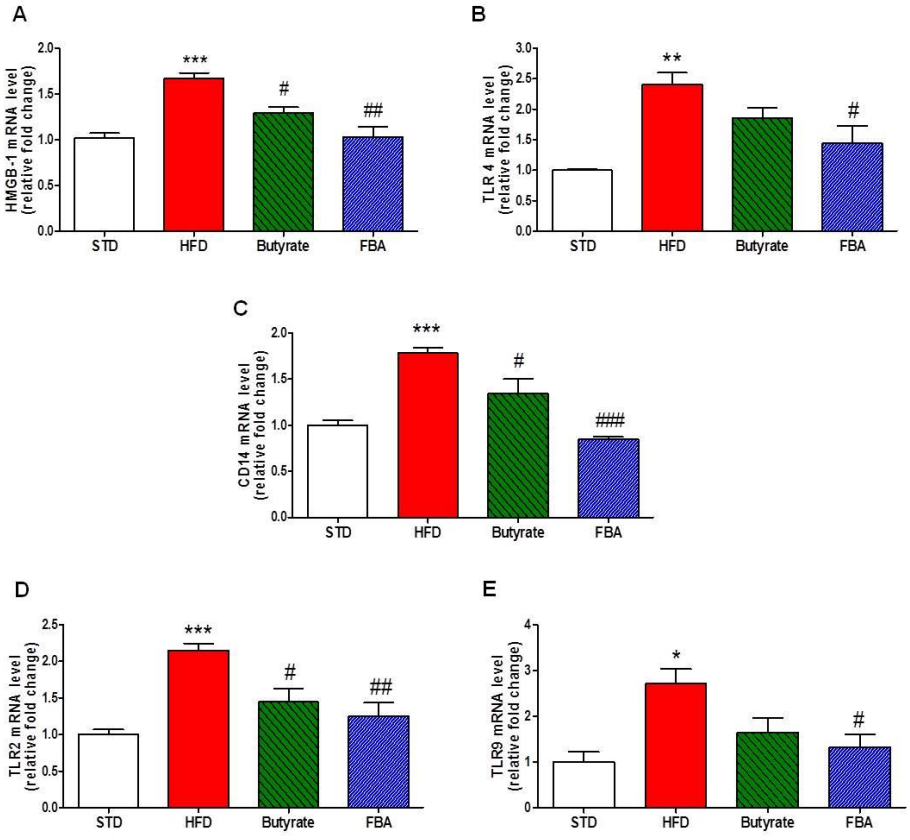


Figure 6.3. Effect of butyrate and FBA on hepatic Toll-like receptors pattern. Panels A and B are the results of real-time PCR for HMGB-1 and TLR4, respectively, in liver extracts from 6 rats on STD, HFD, HFD+butyrate, and HFD+FBA. Panel C shows mRNA expression of coreceptor CD14. TLR2 (D) and TLR9 (E) mRNA levels are shown and expressed as relative fold change to STD. *(P<0.05, **P<0.01 and ***P<0.001 *vs.* STD; # P<0.05, # # P<0.01, # # # P<0.001 *vs.* HFD).

Effect of butyrate and FBA on glucose homeostasis and insulin resistance

To confirm the effects of butyrate and its derivative on insulin resistance in HFD-fed rats, glucose tolerance and insulin signaling were analyzed. In oral glucose tolerance test, blood samples were collected sequentially before, and 30, 60, 90, and 120 min after the glucose load, HFD induced an altered response to glucose at all time points paralleled with STD (**Figure 6.4A and B**): in fact, a marked and significant increase of AUC values was shown in HFD group ($P<0.001$), which was significantly reduced by FBA (**Figure 6.4A and B**). Butyrate and FBA groups exhibited improved response to glucose at all time points, in particular FBA raised a significant reduction of glycemia 90 min after glucose load (**Figure 6.4A**). The insulin receptor is acted on an unique group of regulatory proteins, including PTP1B, which is considered as a major negative regulator of insulin receptor signaling. As depicted in **Figure 6.4C** the HFD feeding determined an over-expression of this enzyme while both treatments showed a significant reduction of this protein in hepatic tissue. Moreover, we showed an increase in SOCS3 protein expression in hepatic tissues from HFD rats, that was significantly inhibited by butyrate and FBA ($P<0.05$, **Figure 6.4D**) confirming the restoration of insulin signaling. According with the inflammatory alterations, other modifications demonstrated metabolic impairment and insulin tissue resistance in HFD fed rats. The basal level of PPAR-α, which regulates fatty acid β-oxidation and catabolism, was detected in liver homogenates from STD fed animals. PPAR-α decreased in liver from HFD rats ($P<0.01$) and restored by butyrate or FBA ($P<0.01$ and $P<0.05$, respectively, **Figure 6.5A**). Very recently, it has been demonstrated that FGF21, a cytokine/hormone that plays an important role in the regulation of lipid and carbohydrate metabolism, was induced by butyrate [531]. In liver extracts, FGF21 was significantly reduced by HFD and its decreased expression was slightly prevented by butyrate and significantly by FBA (**Figure 6.5B**). Moreover, GLUT2, a glucose-sensitive gene in liver cells [534], was markedly reduced by HFD and this effect was significantly prevented by butyrate and FBA (and **Figure 6.5C**). It is well known that adipose tissue, behind its reserve and secretory role, represents an important insulin-sensitive target tissue contributing to glucose homeostasis. Here, the expression of PPAR-γ, a ligand-dependent transcription factor, was lower in HFD compared with STD group, whereas both treatments significantly increased it (**Figure 6.6A**). Consistently, PGC-1α, was significantly reduced in HFD group, while both treatments prevented this effect (**Figure 6.6B**). It is well known that this coactivator controls energy metabolism interacting with severaltranscription factors, including PPAR-α, and its reduction is associated with mitochondrial

dysfunction, reduction in fatty acid oxidation and insulin resistance [535]. Moreover, in adipose tissue butyrate and FBA were also able to restore GLUT-4 protein expression, that was deeply reduced by HFD (P<0.05, respectively, **Figure 6.6C**).

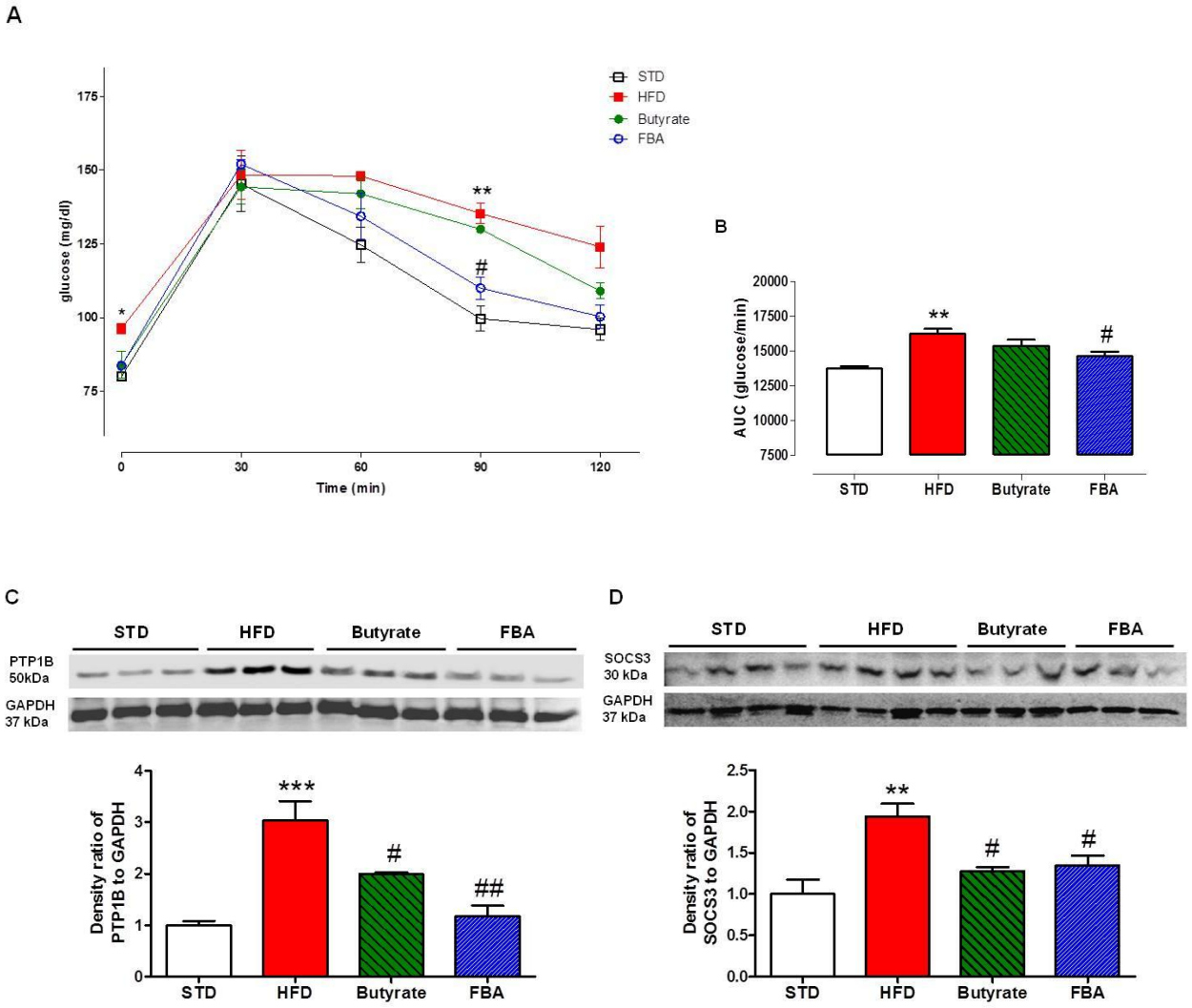


Figure 6.4. Effect of butyrate and FBA on glucose homeostasis and insulin resistance. Glucose tolerance test (A) in STD and HFD-fed rats (n=6, each group) was performed and AUC evaluated (B). Panels C and D show representative Western blot analysis of PTP1B and SOCS3 expression from liver tissue, respectively (n=6 each group). (**P<0.01, ***P<0.001 *vs.* STD; # P<0.05, # # P<0.01 *vs.* HFD).

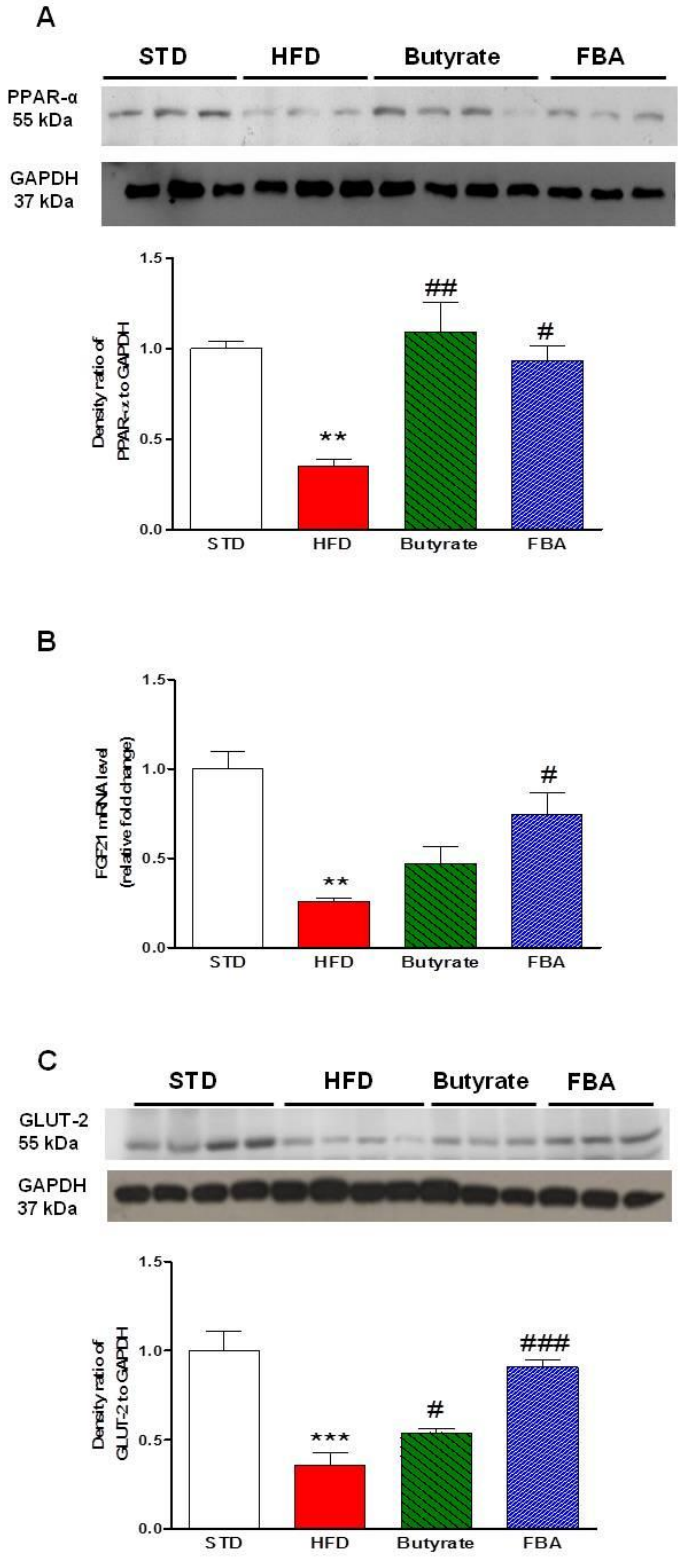


Figure 6.5. Effect of butyrate and FBA on metabolic impairment and insulin resistance in liver from HFD fed rats. Panel A shows a Western blot analysis of PPAR-α expression (n=6 each group). FGF21 mRNA abundance (B) and GLUT-2 protein expression level (C) in the liver extracts were quantified by real-time PCR and Western blot analysis, respectively (n=6 each group). (**P<0.01, ***P<0.001 *vs.* STD; # P<0.05, # # P<0.01, # # # P<0.001 *vs.* HFD).

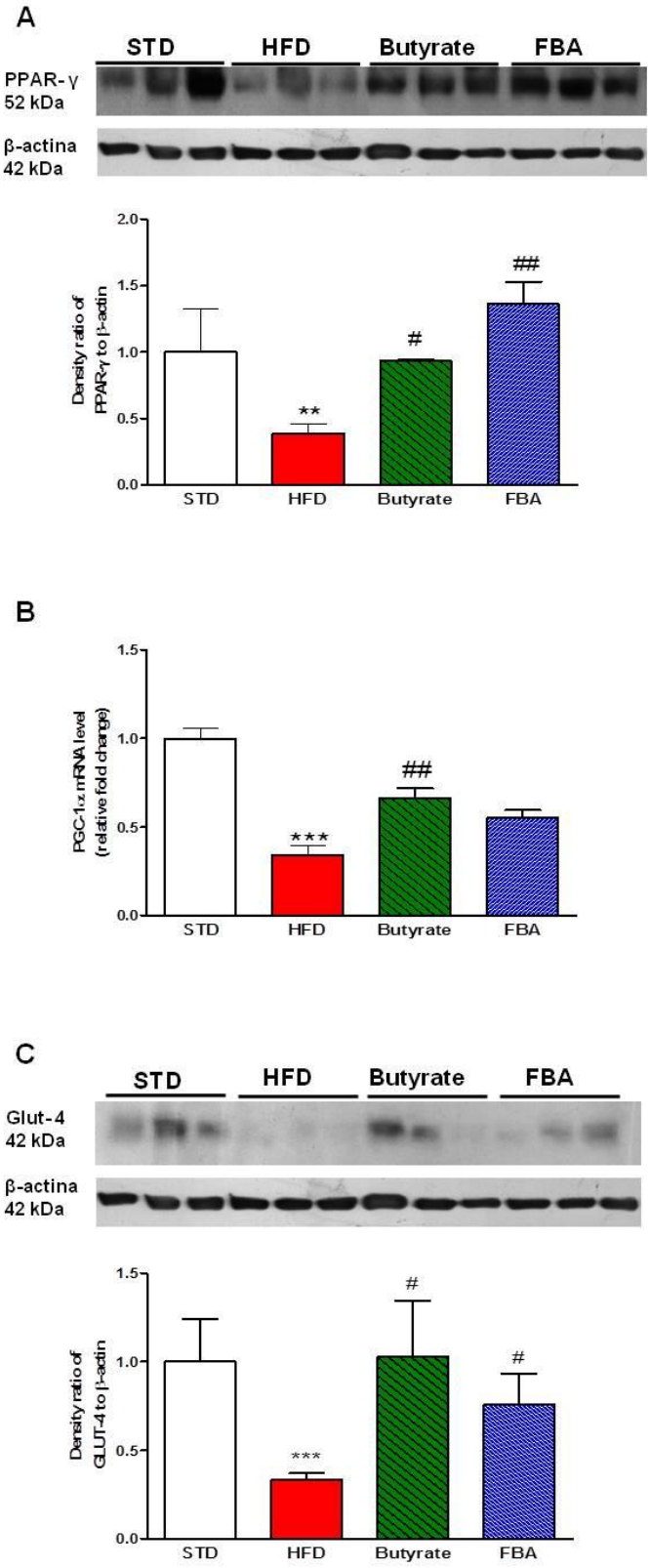


Figure 6.6. Butyrate and FBA are able to modulate metabolic proteins in adipose tissue. Representative Western blot of PPAR-γ (A) is shown (n=5 each group). Panel B shows real-time PCR results from PGC-1α mRNA levels (relative expression toSTD) in adipose tissue (n=6 each group). (C) Western blot analysis of GLUT-4 protein expression in adipose tissue extracts is shown (n=5 each group). (**P<0.01 and ***P<0.001 *vs.* STD; # P<0.05, # # P<0.01 *vs.* HFD)**.**

6.4 Discussion

Metabolic and anti-inflammatory effects of butyrate and its derivative, phenylalanine-butyramide (FBA), were examined in this study in a HFD-induced rat model of hepatic steatosis and insulin resistance. Sodium butyrate shows considerable drawbacks: it has fairly strong hygroscopicity and butirric nauseating smell, associated to a poor compliance related to extremely unpleasant taste and epigastric discomfort after oral assumption. On the other hand, the conjugate of butyrate to phenylalanine, FBA, has allowed to obtain a molecule with chemical-physical characteristics suitable for an easier oral administration compared to butyrate. The most important observation is that both treatments prevented the impairment of glucose homeostasis and the development of insulin resistance. In our model there are two major hallmarks of insulin resistance: hepatic inflammatory process and alteration of glucose tolerance related to an impairment of insulin signaling. The improvement in insulin sensitivity may be, at least in part, a consequence of anti-inflammatory effects of these compounds in this nutritional model. Insulin controls whole body glucose homeostasis with several mechanisms, such as the promotion of glucose disposal in sensitive target tissues, such as liver and fat. Our study shows that both treatments are able to prevent liver inflammation and damage, steatosis, the onset of IR, and imbalance of TLRs pattern in the early stage of NAFLD. In particular, we showed a reduction in hepatic lipid accumulation mainly significant in FBA treated rats, together with a reduction of the inflammatory infiltrates. The evaluation of the fibrotic process revealed no appreciable modification in HFD fed rats, and consequently no modification by both butyric treatments (data not shown), even if TGF-β, an early pro-fibrogenic marker, was up-regulated in liver from rats on HFD, and markedly reduced by both treatments. TGF-β is considered the most powerful mediator of hepatic stellate cell activation and kupffer cells are a main source of TGF-β in the liver, promoting, collectively to inflammatory cytokines and oxidative stress, later fibrosis [536].

Moreover, both treatments normalize the hepatic markers of steatosis, and preserve glucose tolerance, reducing fasting glucose and modulating HOMA-IR. It is now clear that TNF-α and IL-6 represent crucial effectors of IR, that link liver inflammatory process to hormonal and metabolic alterations [93,506]. In our experimental model, both compounds reduce the above reported cytokines in parallel with a lower expression of PTP1B and SOCS3 inhibitory proteins, suggesting two molecular mechanisms of insulin sensitization. In accordance, recently it has been highlighted how the prototypic phoshotyrosine-specific phoshatase PTP1B dephosphorylates the insulin receptor and downstream IRS-1/2 proteins. Its inhibition enhances insulin signaling and attenuates

insulin resistance both in conventional and non conventional insulin responsive tissues [537], indicating PTP1B as a target for the development of novel therapeutics for diabetes and obesity. On the other hand, SOCS family, including SOCS3, associates with the insulin receptor and inhibits its signaling through ubiquitin-induced degradation of IRS-1. In particular, the induction of SOCS3 in liver may be an important mechanism of IL-6-mediated insulin resistance [93]. Moreover, both treatments reduce the activation of NF-κB pathway induced by HFD, reducing IκBα degradation and inhibiting p50, with a subsequent reduction not only in cytokine transcription (TNF-α, IL-6 and IL-1β), but also in inflammatory enzymes (COX-2 and iNOS). Furthermore, we found that HFD increases macrophage infiltration markers, MCP-1 and F4/80, and both butyrate treatments are able to reduce these parameters, in particular reducing F4/80-positive macrophage, indicative of M1state, associated with inflammation and tissue injury [538].

Here, we demonstrate that both treatments reduce inflammation and its mediators inhibiting NF-κB activation arguably through the down-regulation of TLR pattern in the liver, which are involved in bacterial sensing and are crucial for "liver tolerance". We demonstrate that FBA and, to a lesser extent, butyrate are able to limit the increased transcription and expression of TLRs induced by HFD. The increased expression of hepatic TLRs confirms a greater exposure of the liver to ligands for these receptors deriving from the intestine (i.e. pathogen-associated molecular patterns or PAMPs and endogenous damage-associated molecular patters or DAMPs), including the widely expressed nucleus protein HMGB1. Recently, it has been hypothesized that FFA-related HMGB1 release mediates the activation of TLR4 signaling in hepatocytes and plays an essential part in the early stage of NAFLD induced by HFD [523]. Here, hepatic HMGB1 transcription was strongly up-regulated by HFD feeding while both butyrate, and in particular, FBA determined a significant reduction of its levels. In parallel, they also normalized the expression of TLR4 and its co-receptor CD14 in liver. Accordingly, we evidenced the same profile of activity of butyric treatments on TLR2 mRNA. Consistently with our data, Ehses et al., [525] have reported that TLR2 deficient mice are protected from IR and β cell dysfunction induced by HFD, linking TLR2 to the increased dietary lipid and the alteration of glucose homeostasis. Finally, FBA significantly inhibits the HFD-related increase in TLR9 synthesis. Intracellular TLR9 activates innate immune defenses against viral and bacterial infection and plays a role in the pathogenesis of NASH [118]. We previously demonstrated that HFD feeding is associated with the reduction of PPAR-α expression in liver [70] and the administration of a PPAR-α agonist or probiotics restores PPAR-α and improves hepatic steatosis [70,71]. Here, we evidence that butyrate and FBA are also able to do this. Our data confirm recent in vitro and in vivo findings that identify butyrate as a new inducer of FGF21 [531].

In that study, butyrate injection increased FGF21 serum concentration and protein levels in the liver of obese mice through activation of PPAR-α which was dependent on HDAC3 inhibition by butyrate. Despite the differences in the two experimental models (6 weeks *vs.* 20 weeks of diet, an sub-chronic treatment vs an acute one), in our experiments, butyrate and its derivative are still able to restore hormone levels in the early stage of NAFLD, in liver of non obese rats assuming the involvement of HDAC3 inhibition. FGF21 is a metabolic hormone predominantly produced by the liver, but also expressed in adipocytes and pancreas, where it regulates glucose and lipid metabolism through pleiotropic actions [512]. We studied PPAR-α and PPAR-γ expression in tissues where they are more abundant, liver and adipose tissue, respectively [539]. PPAR-γ promotes fatty acid uptake and increases insulin sensitivity by upregulating GLUT-4, an insulin dependent glucose transporter in adipose tissue and striated muscle [515] and attenuating the induction of SOCS3 [516]. In the current report, we provide evidence that butyrate and FBA not only prevent, in adipose tissue, the HFD-induced reduction of PPAR-γ but also positively modulate PPAR-γ-coactivator PGC-1α [540]. This transcription coactivator controls energy metabolism, interacting with several transcription factors, including PPAR-α and PPAR-δ, that regulate gene transcription for mitochondrial biogenesis and respiration [541]. In fact, the reduction in PGC-1α function is associated with mitochondrial dysfunction, reduction in fatty acid oxidation, and risk for IR or type 2 diabetes [542]. Dietary intervention of PGC-1α activity holds promise in the prevention and treatment of metabolic syndrome [477,543]. Our data support the concept that the stimulation of PGC-1α activity may be a molecular mechanism of butyrate activity, in agreement with previous data demonstrating that the inhibition of histone deacetylases and activation of AMPK may contribute to the PGC-1α regulation [477]. Consistently, butyrate and FBA are able to normalize the facilitative hexose transporter, GLUT-2 and insulin-stimulated glucose transport GLUT-4 expression in liver and adipose tissue, respectively, supporting systemic effects of both compounds. Our data are in agreement with previous findings demonstrating that butyrate up-regulates GLUT-2 mRNA abundance in other cell types, such as Caco2-BBe monolayers, by activating specific regions within the human GLUT2 promoter [544]. GLUT-2 is located in the plasma membrane of hepatocytes and pancreatic beta cells, in contrast with GLUT-1 and GLUT-3, has a low affinity for glucose. Their high Km (15-20 mM) allows for glucose sensing; rate of glucose entry is proportional to blood glucose levels. GLUT-4 transporters are insulin sensitive, and are found mainly in muscle and adipose tissue. As muscle is a principle storage site for glucose and adipose tissue for triglyceride (into which glucose can be converted for storage), GLUT-4 is important in post-prandial uptake of excess glucose from the bloodstream or in other conditions of over-nutrition

(i.e. HFD). Moreover, recent findings have reported an improvement of liver glycogen storage by acute butyrate supply that was explained by the competition between butyrate and glucose oxidation, and by a likely reduced glycogenolysis from the newly synthesized glycogen [545].

6.5 Conclusions

To our knowledge, our work is the first to propose concomitant biochemical mechanisms in the liver and adipose tissue to better understand how butyrate and its derivative may regulate glucose metabolism and improve insulin sensitivity. Our results show a protective effect of butyrate to limit early molecular events underlying IR linked to steatosis, suggesting a potential clinical utility as innovative, preventive and therapeutic strategy for NAFLD. In fact, these treatments prevent the transition from steatosis toward steatohepatitis, dampening the onset of several hits responsible for the shift and the progression of the disease. Since FBA does not have the characteristic odor of rancid butyrate, this derivative may represent a viable therapeutic alternative to butyrate, favoring a better compliance and a greater effectiveness.

CHAPTER 7: EFFECTS OF A SYNBIOTIC-BASED LACTOBACILLUS PARACASEI B21060 ON EPITHELIAL BARRIER FUNCTION AND TISSUE REPAIR IN DEXTRAN SODIUM SULFATE-INDUCED COLITIS IN MICE.

7.1 Introduction

IBDs are thought to result from inappropriate and ongoing activation of the mucosal immune system driven by penetration of normal luminal flora through defective tight-junction proteins (TJ). The altered TJ structure in ulcerative colitis results in impaired barrier function, which may lead to increased uptake of luminal antigens and/or adjuvants that overwhelm the net suppressive tone of the mucosal immune system [546]. Commensal bacteria regulate key epithelial cell functions that contribute and maintain intestinal epithelial barrier integrity against injury [547]. However, the precise innate immune mechanisms of beneficial host-microbial interactions are not yet fully understood [548]. The intestinal epithelium is the interface for the interaction between gut microbiota and host tissues [549]. This barrier is enhanced by the presence of a mucus layer and immune factors that are produced by the host [550]. Antimicrobial peptides for innate immunity are produced by Paneth cells (e.g., α-defensins, lysozyme C, phospholipases, and C-type lectin, primarily regenerating islet derived 3-gamma, RegIIIγ) or enterocytes (RegIIIγ) [551,552]. Adaptive immune system effectors that are secreted into the intestinal lumen, such as IgA, may also restrict bacterial penetration into the host mucus and mucosal tissue [553]. These immune factors allow the host to control its interactions with gut microbiota and shape its microbial communities [552]. The development of immune-modulating probiotic therapeutic approaches for IBD is related to observations that several invasive pathogenic bacteria including *E. coli* and *C. Difficile* are pro-inflammatory and associated with disease development in patients [554,555]. In contrast, bifidobacteria are reported to be deficient in rectal biopsies from patients with IBD [556] and studies of faecal flora in patients with Crohn's disease suggest a deficiency in both lactobacilli and bifidobacteria [557]. Therefore, in IBD patients there are respectively reduced and increased levels of commensal bacteria and pathogenic bacteria. The most widely used probiotics are Bifidobacterium and Lactobacillus species, both of which have been tested in clinical trials of

irritable bowel syndrome [558]. Overall, human IBD trials to date suggest that probiotics display no overt side effects, but conflicting reports on probiotic efficacy highlight the importance of selecting well-characterised probiotic strains and in delivering intact pharmaceutical formulations at an appropriate dose level to the inflamed regions of the intestine [559]. Testing of probiotics in mouse models of IBD has yielded encouraging data that have prompted human trials. The distal intestine of humans contains tens of trillions of microbes: this community (microbiota) is dominated by members of the domain Bacteria but also includes members of Archaea and Eukarya, and their viruses. The vast repertoire of microbial genes (microbiome) present in the distal gut microbiota performs myriad functions that benefit the host [560]. The mucosal immune system co-evolves with the microbiota beginning at birth, acquiring the capacity to tolerate components of the microbial community while maintaining the capacity to respond to invading pathogens. The gut epithelium and its overlying mucus provide a physical barrier. Epithelial cell lineages, notably the Paneth cell, sense bacterial products through receptors for microbe-associated molecular patterns (MAMPs), resulting in regulated production of bactericidal molecules [561]. Mononuclear phagocytes continuously survey luminal contents and participate in maintenance of tissue integrity, and the initiation of immune responses [562,563]. Several families of innate receptors expressed by hematopoietic and non-hematopoietic cells are involved in recognition of MAMPs, such as Toll-like receptors, nucleotide binding oligomerization-domain protein-like receptors (NLRs), and C-type lectin receptors [564,565]. Recently, the NLRP sub-family of the NLR family of proteins, distinguished from the other NLRs by an N-terminal pyrin domain, has been implicated in the pathogenesis of chronic inflammatory conditions [566,567]. Inflammasomes are cytoplasmic multi-protein complexes composed of one of several NLR proteins, including NLRP1, NLRP3, and NLRC4, that function as sensors of endogenous or exogenous stress or damage-associated molecular patterns [568]. Upon sensing the relevant signal, they assemble, typically together with the adaptor protein, apoptosis-associated speck-like protein (ASC), into a multi-protein complex that governs caspase-1 activation and subsequent cleavage of effector pro-inflammatory cytokines including pro-IL-1β and pro-IL-18 [565,569]. Several other members of the NLR family, including NLRP6 and NLRP12, possess the structural motifs of molecular sensors, and are recruited to the "specks" formed in the cytosol by ASC oligomerization, leading to pro-caspase-1 activation [570]. Given these considerations, a specific aim of this study was to investigate whether in a mouse models of ulcerative colitis, the synbiotic formulation Flortec based on *Lactobacillus paracasei B21060* could prevent or repair the damage inuced by sodium dextran sulphate. Drugs that can decrease epithelial permeability through closing epithelial tight junctions are currently the focus of

intense research efforts [571]. Thus, a key question posed in the current study was whether this probiotic strain could preserve innate immune barrier equilibrium through TJ regulation, which balances mucosal homeostasis against inflammatory stress-induced damage in mice. Besides, in order to investigate how this synbiotic regulate the epithelial repair process, we sought to explore the mechanism that requires an inflammasome, involving NLRP6, NLRP3 and caspase-1, and leads to the cleavage of pro-IL-18 in our model of colitis. Our data showed significant curative effects of this synbiotic formulation in DSS model of colitis and suggests not only a potential therapeutic role for this agent in this pathology, but also the possibility that a supplement of these lactobacilli might prevent the relapse of UC.

7.2 Materials and Methods

DSS-induced colitis and animal treatments

Experimental colitis was induced in ten weeks old BALB/c male mice (25±2 g) (Harlan-Corezzano, Italy) by 2.5% DSS (wt/vol) (MP Biomedicals, Irvine, CA) in sterile drinking water *ad libitum* for five days, followed by drinking water without DSS for twelve days. Mice were randomly divided into four groups (n=10 each group) as following: 1. a control animals (CON), 2. DSS treated mice (DSS), 3. DSS mice treated with Flortec as preventive therapy (FLO PREV) and 4. DSS mice treated with Flortec as curative therapy (FLO CUR). Flortec (Bracco, Milan, Italy) is a synbiotic formulation containing viable lyophilized *Lactobacillus paracasei B21060* mixed with prebiotics fructo-oligosaccharides and arabinogalactan. This formulation was available as powder and dispensed in 6 g bag containing about 2.5×10^9 CFU of the bacteria. Daily synbiotic treatment (*L. paracasei B21060* $2.5x10^7$ bacteria/10g bw; fructo-oligosaccarides 7mg/10g bw, and arabinogalactan 5mg/10g bw by gavage) started seven days before (PREV) or two days after (CUR) DSS challenge. DSS group without synbiotic formulation, received H_2O by gavage as drug's vehicle. Colitis was assessed by the daily monitoring of body weight, stool consistency and fecal blood. All procedures involving animals were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 of Ministero della Salute and associated guidelines of the European Communities Council Directive of November 24, 1986 (86/609/ECC). Prior to sample collection, animals, kept overnight fasted, were euthanized by an isoflurane anesthesia, followed by cervical dislocation to minimize pain. All efforts were made to minimize animal suffering. Blood samples from animals were collected by cardiac puncture and serum obtained. At 19^{th} days mice were killed and full intestine and colons measured. Colon tissue was excised and immediately frozen. Segments of colon were assessed histologically by hematoxylin and eosin staining. In **figure 7.0** the scheme of experimental protocol and animal treatments are summarized.

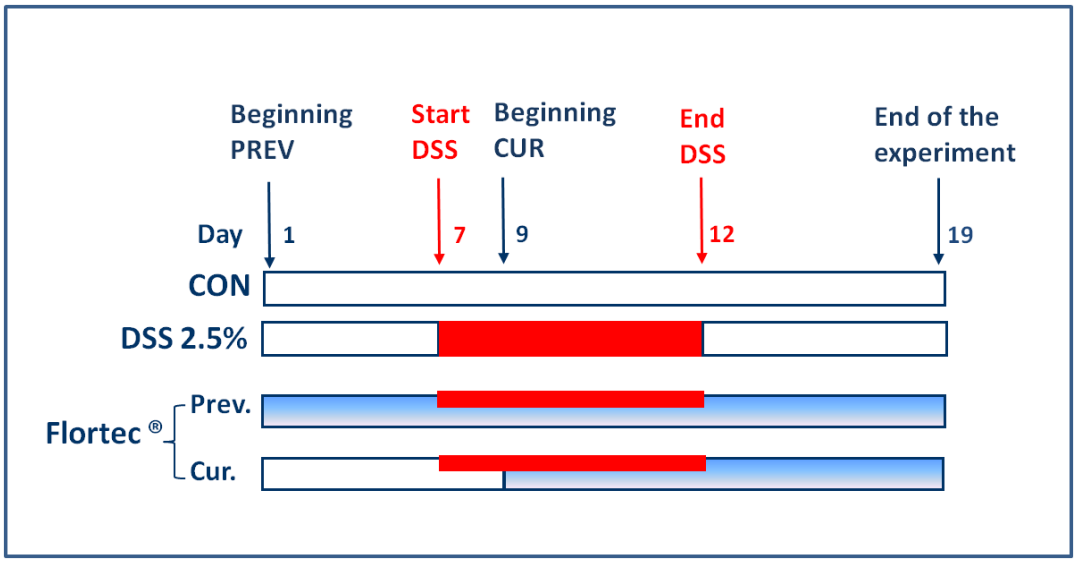


Figure 7.0. Experimental protocol used for this study

Evaluation of experimental colitis

In all animals, weight, presence of blood and stool consistency were determined daily as previously described [572]. Disease activity index (DAI) was determined by combining scores of a) weight loss b) stool consistency and c) bleeding (divided by 3). Each score was determined as follows, change in weight (0:<1%, 1: 1–5%, 2: 5–10%, 4:>15%), stool blood (0: negative, 2: positive) or gross bleeding (4), and stool consistency (0: normal, 2: loose stools, 4: diarrhea) as previously described [573]. Body weight loss was calculated as the percent difference between the original body weight and the actual body weight on any particular day. Typically in DSS colitis animals will lose 10–15% body weight over the course of 10 days. The appearance of diarrhea is defined as mucus/fecal material adherent to anal fur. The presence or absence of diarrhea was scored as either 1 or 0, respectively, and the cumulative score for diarrhea was calculated by adding the score for each day and dividing by the number of days of exposure. Rectal bleeding was defined as diarrhea containing visible blood/mucus or gross rectal bleeding and scored as described for diarrhea.

Western Blotting

Segments of colon tissue were homogenized on ice in lysis buffer (Tris-HCl, 20 mM pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM

Na_3VO_4, 10 μg/ml leupeptin and trypsin inhibitor). After 1 h, tissue lysates were obtained by centrifugation at 21000xg for 15 min at 4°C. The protein concentration of the samples was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Segrate, Milan, Italy), using bovine serum albumin as the standard. For Western blot analysis, tissue lysate was dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to SDS-PAGE. The blot was performed by transferring proteins from a slab gel to nitrocellulose membrane at 264 mA for 45 min at room temperature. The filter was then blocked with 1x PBS, 5% non fat dried milk for 40 min at room temperature and probed with rabbit polyclonal antibody anti-3-nitrotyrosine (1:8000; Millipore, Billerica, MA USA) dissolved in 1x PBS, 5% non fat dried milk, 0.1% Tween 20 at 4°C, overnight. The secondary antibody was incubated for 1 h at room temperature. Subsequently, the blot was extensively washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, Piscataway, NJ, USA). The optical density of the bands was determined by a GS-800 imaging densitometer (Bio-Rad). Western blot for GAPDH (Sigma-Aldrich; Milan Italy) was performed to ensure equal sample loading.

MDA measurement

MDA levels in colon were determined as an indicator of lipid peroxidation [574]. Tissues were homogenized in 1.15% KCl solution. An aliquot (200 μl) of the homogenate was added to a reaction mixture containing 200 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid, and 600 ml of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000xg for 10 min. The supernatant absorbance was measured at 550 nm by spectrophotometry and the concentration of MDA was expressed as μmol MDA/mg protein of tissue homogenate. A standard curve was prepared using MDA bisdimethylacetal as the source of MDA. All solutions were freshly prepared on the day of assaying.

Measurement of MPO activity

Proximal colonic tissues were homogenized twice for 30s at 4°C in 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM sodium phosphate, pH 6.0, according to Bradley et al., [575]. The homogenates were clarified by centrifugation at 13000xg for 15 min, at 4°C, and were assessed for MPO (Myeloperoxidase) activity in 3 ml 50 mM sodium phosphate, pH 6.0, containing 16 mM aqueous guaiacol and 5.9 mM H_2O_2, as previously described [576]. The

increase in absorbance was measured for 2 min at 470 nm using The iMark microplate absorbance reader (Bio-Rad). Protein concentrations were determined using the Bio-Rad protein assay. MPO activities were expressed in U.MPO/mg protein with 1 U hydrolyzing 1 μmol H_2O_2/min.

Real-time semi-quantitative PCR

Total RNA isolated from colon was extracted using TRIzol Reagent (Invitrogen Biotechnologies), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 μg total RNA. PCRs were performed with Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories). The primer sequences are reported in **Table 7.1**. The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing extension at 60°C for 60 s. Each sample contained 1-100 ng cDNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/l of each primer (Eurofins MWG Operon; Huntsville, AL, USA). in a final volume of 25 μl. The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

Table 7.1. Real-Time PCR Primer Sequence

Target gene	Forward primer (5'→3')	Reverse primer (3'→5')	Accession Number
Casp1	TGGTGTTGAAGAGCAGAAAGC	GCCCAGAGCACAAGACTTCTGAC	NM_009807.2
CCL5	AGATCTCTGCAGCTGCCCTCA	GGAGCACTTGCTGCTGGTGTAG	NM_013653.3
COX-2	TGTGACTGTACCCGGACTGG	GGGTGAACCCAGGTCCTCGCTT	NM_011198.3
Defb-1	GGTGTTGGCATTCTCACAAG	ACAAGCCATCGCTCGTCCTTTATG	NM_007843.3
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	NM_017008 XM_216453
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	NM_010548.2
IL-18	CAGGCCTGACATCTTCTGCAAC	CTGACATGGCAGCCATTGT	NM_008360.1
MUC-1	TCGTCTATTTCCTTGCCCTG	ATTACCTGCCGAAACCTCCT	NM_013605.2
NE (Elane)	CCTTCTCTGTGCAGCGGATCTTC	ACATGGAGTTCTGTCACCCAC	NM_015779.2
NLRP3	TGCTCTTCACTGCTATCAAGCCCT	ACAAGCCTTTGCTCCAGACCCTAT	NM_145827.3
NLRP6	CTGAGACTGGTGAGCTGTGGA	ATTGCCTCACAGAGTGGACG	NM_133946.2
Occludin	ATGTCCGGCCGATGCTCTCTC	CTTTGGCTGCTCTTGGGTCTGTAT	NM_008756.2
TNF-α	CATCTTCTCAAAACTCGAGTGACAA	TGGGAGTAGATAAGGTACAGCCC	NM_012675.3
ZO-1	ACCCGAAACTGATGCTGTGGATAGA	AAATGGCCGGGCAGAACTTGTGTA	NM_001163574.1

Immunofluorescence analysis of occludin and zonula occludens (ZO)-1

Colon segments were immediately removed, washed with PBS, mounted in embedding medium (Pelco Cryo-Z-T, Ted Pella inc, Redding, California), and stored at -80 °C until use. Cryosections (7 μm) were fixed in formaldehyde 2%+PBS at RT for 10 min for occludin or in methanol for 10 min at RT for ZO-1. Non-specific background was blocked by incubation with normal goat serum in PBS and 0.1% Triton X-100. Sections were incubated for 2 h with rabbit anti-occludin (1:50 for occludin, Santa Cruz Biotechnology, Inc.) or rabbit anti-ZO-1 (1:100 for ZO-1, Invitrogen corporation, Camarillo, California, USA). Sections were probed with secondary antibodies goat anti-rabbit Alexa Fluor® 488 for ZO-1 and goat anti-rabbit Alexa Fluor® 594 for

occludin (1:200, Invitrogen corporation). Slides were mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, California, USA), and visualized on a fluorescence microscope using a 640 objective, and images were stored digitally with Leica software. Two negative controls were used: slides incubated with or without primary antibody. All the staining were performed in duplicate in non-serial distant sections, and analyzed in a double-blind manner by two different investigators.

Serum Adiponectin detection

Whole blood taken through cardiac puncture was centrifuaged after 24h at 1500xg at 4°C for 15 min. So serum obtained was stored at -80°C and then used for adiponectin levels detection by Enzyme Linked Immuno Sorbent Assay (ELISA) (Quantikine® Immunoassay, RD & SISTEMS, Minneapolis, MN) following manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by ANOVA test for multiple comparisons followed by Bonferroni's test, using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA). Statistical significance was set at $P<0.05$.

7.3 Results

Flortec reduces mice susceptibility to DSS-induced colitis

During all experimental time body weight was monitored every day. As depicted in weight change graph (**Fig. 7.1A**), DSS-challenged mice treated with vehicle showed a reduction of weight, which becames significant from 15^{th} day until end of experimental period (18^{th} day) ($P<0.05$ *vs.* CON). Both preventive and curative treatment with Flortec preserved from this body weight loss, showing so a beneficial effect on animal's gain and health. This protective action of synbiotic was more evident by area under curve (AUC) analysis (**Fig. 7.1A**). Assessment of disease activity index (DAI) after 7 days from DSS end, reveals in DSS-challenged mice plus vheicle, a strong increase of colitis gravity (**$P<0.01$ *vs.* CON). Instead both therapeutic schemes with synbiotic (PREV and CUR) significantly prevented the development of ulcerative colitis manifestations (**Fig. 7.1B**) (## $P<0.01$ *vs*. DSS).

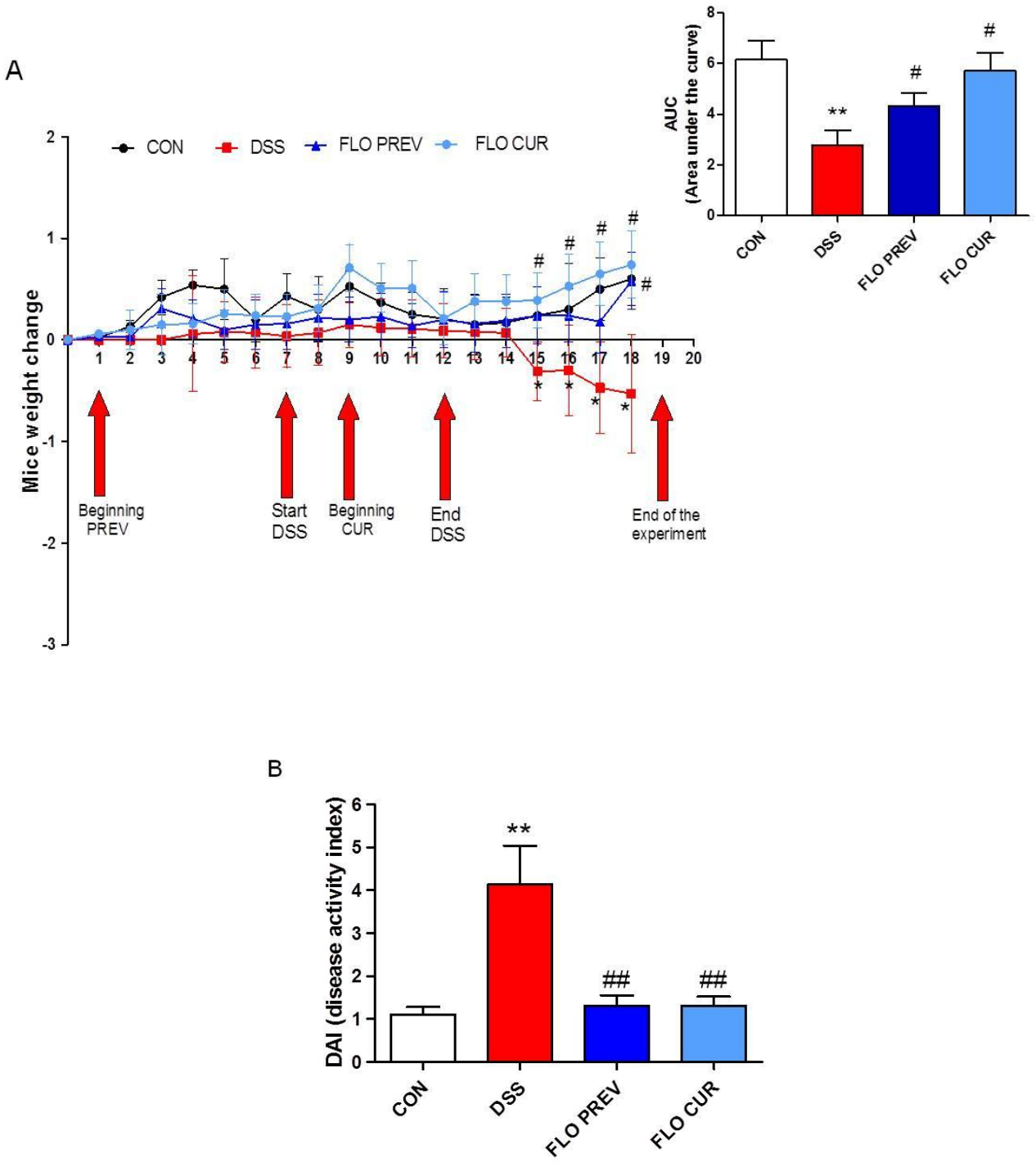


Fig. 7.1 Effects of synbiotic on induction and recovery from DSS induced colitis. Mice were treated with 2.5% DSS in the drinking water for 5 days and then returned to normal drinking water for an additional 7 days. (A) Changes in body weight and (B) DAI values on day 12. Data are mean ± SD from eight mice/group.

Flortec improves tissue histopatholgy and prevents colon damage

Administration of Flortec significantly ameliorated all clinical signs of DSS-induced colonic inflammation (weight loss, rectal bleeding, and mortality), whereas DSS-control mice showed marked colitis-associated signs. Histological examination of the distal part of DSS-alone colons revealed hemorrhagic walls with multiple ulcerations, mucosal edema, and large hyperplasia of the mucosa-associated lymphoid tissue (MALT). DSS mice treated with Flortec had intact colonic epithelium, no erosions, and rarely inflammatory infiltrates in the lamina propria (**Fig. 7.2A and C**). Both therapeutic schemes (PREV and CUR), significantly restored DSS induced colon shortening (**Fig. 7.2B**) (***P<0.001 *vs.* CON; ## P<0.01 *vs.* DSS).

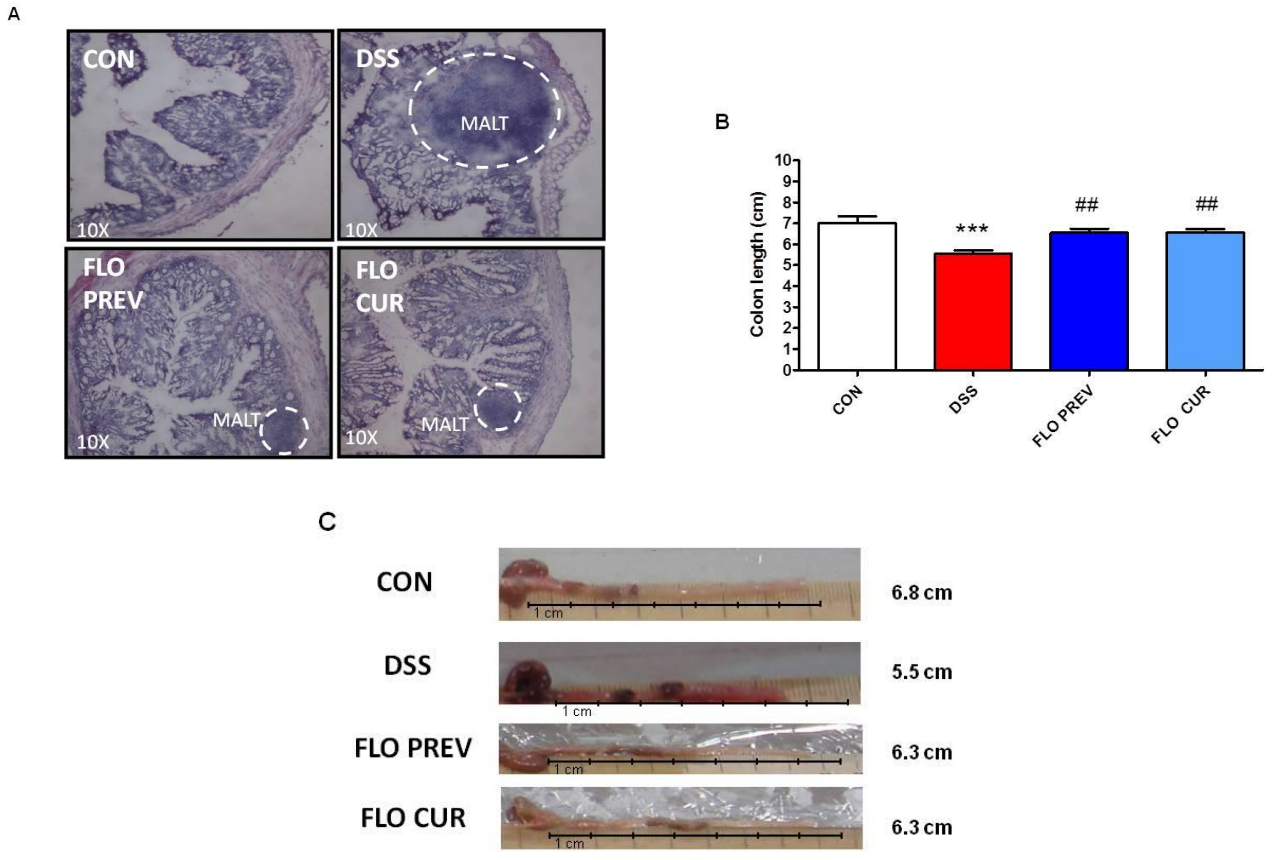
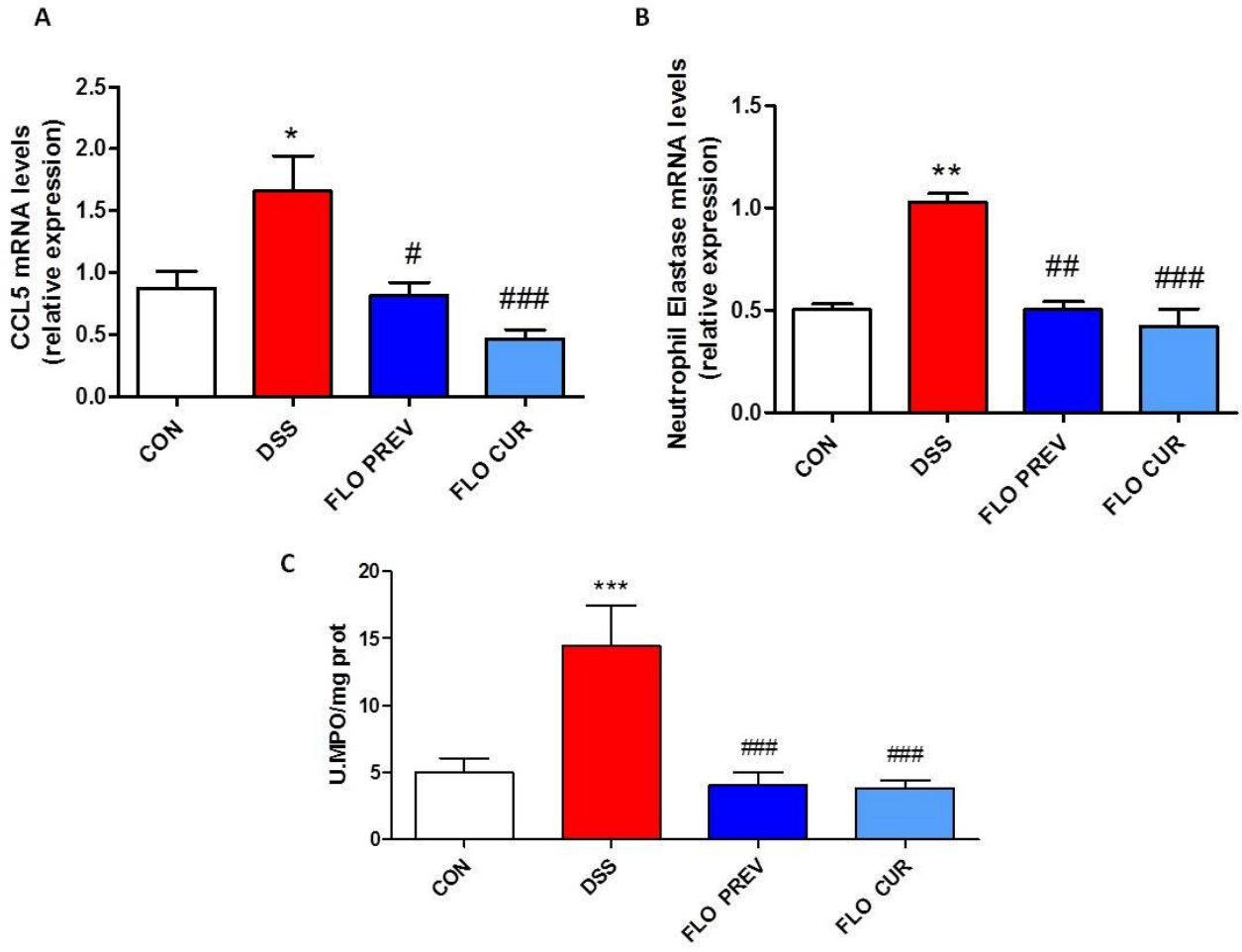


Fig. 7.2 Effects of Flortec on acute DSS-induced colitis. (A) Representative H&E-stained section of colons from synbiotic or DSS+vehicle-treated mice (Scale bar = 200 µm, magnification 100X). (B) Colon length measurement and (C) representative colon images of control mice and DSS-challenged mice treated or untreated with synbiotic. Data are mean ± SEM from 7 to 8 mice/group. H&E images are representative of 5 slides for each group.

Flortec reduces DSS-induced CCL5 production and inflammatory immune cell recruitment in colon tissue

Analysis of chemokine mRNA levels by Real-Time PCR, showed a significant up-regulation of Chemokine (C-C motif) ligand 5 (also CCL5) (**Fig. 7.3A**) and Neutrophil Elastase (NE) (**Fig. 7.3B**) in colon tissue from DSS mice (*P<0.05; **P<0.01 *vs*. CON). The immune cell recruitment was also evidenced by high levels of MPO (**Fig. 7.3C**), nitrosylated proteins **(Fig. 7.3D**) and MDA amount (**Fig. 7.3E**) revealed in these animals. The synbiotic treatment was able to significantly reduce all these parameters (#P<0.05; P<0.01; P<0.001 *vs.* DSS), exhibiting so an effect not only on immune cells trafficking but also on inflammatory status in colonic mucosa (**Fig. 7.3A-E**).



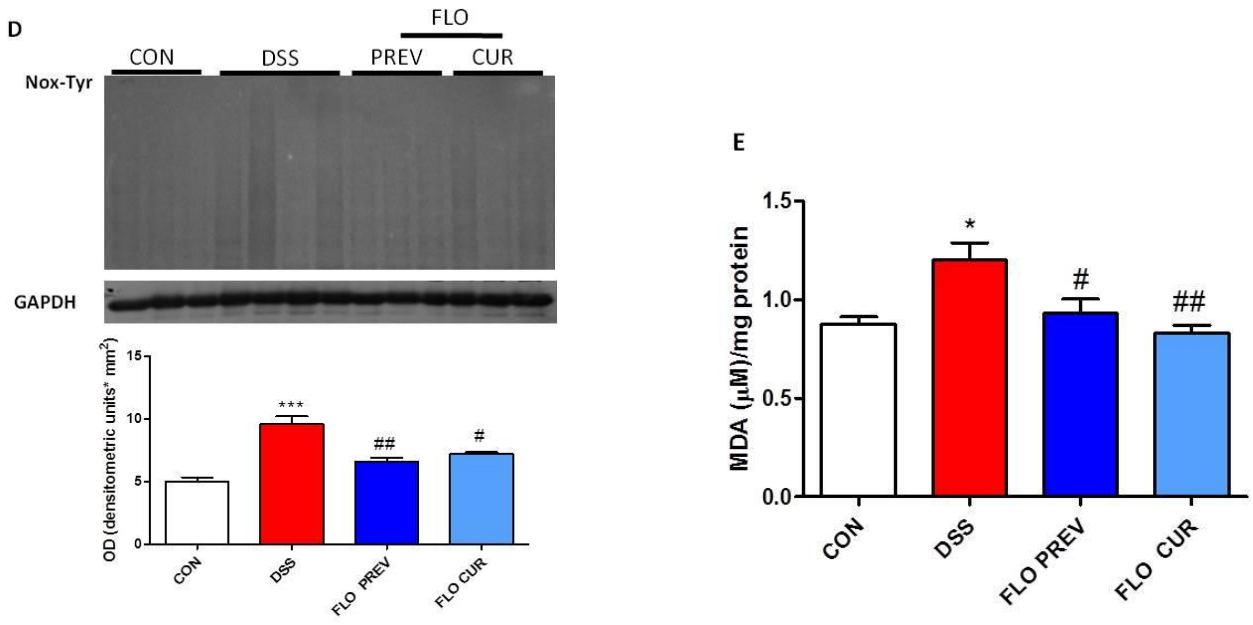


Fig. 7.3. DSS induces leukocytes infiltration and ROS damage in colon tissue. (A) mRNA levels of chemokine (C-C motif) ligand 5 (CCL5) and (B) Neutrophil elastase. (C) MPO activity measurement in proximal colon section. (D) Nitrosylated proteins levels and MDA production assessment in colon from DSS-challenged mice treated or untreated with synbiotic as preventive or curative protocol. Data are mean ± SEM from 7 to 8 mice/group.

Effects of Flortec on pro- and anti-inflammatory parameters and adipokines after DSS damage

After 5 days exposure to DSS followed by 7 days of recovery, there was a significant (P<0.001 *vs.* CON) increase in TNF-α and COX-2 mRNA levels in the colons of DSS-challenged mice relative to control group (**Fig. 7.4A-B**). Additionally, downregulation in colon levels of mRNA IL-10 (**Fig. 7.4C**) and in serum levels of adiponectin (**Fig. 7.4D**), following exposure to DSS, were also evidenced in these animals. Treatment of mice for 19 days (PREV) or 12 days (CUR) with synbiotic, reduced pro-inflammatory cytokines and enzymes (TNF-α and COX-2 respectively), and restored regulatory cytokines (IL-10) or adipokine (adiponectin) levels in colon tissue and serum respectively (#P<0.05; #P<0.01; P<0.001 *vs*. DSS).

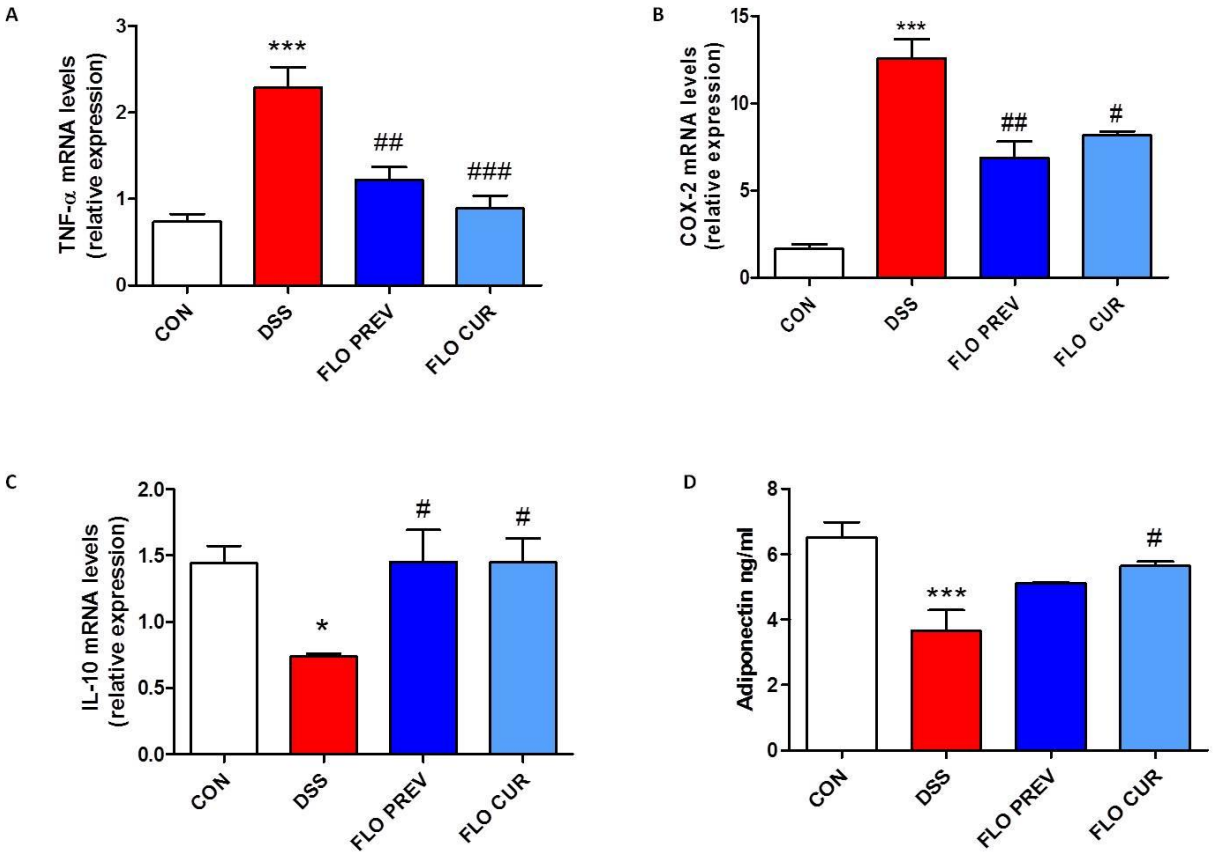


Fig. 7.4. Pro- and anti-inflammatory mediators production in colonic mucosa and serum. DSS can increase the production significantly of pro-inflammatory mediators TNF-α (A) and COX-2 (B) in colon tissue, and reduce transcriptional levels of anti-inflammatory mediators in colon (C) and serum (D). Data are mean ±SEM from 7 to 8 mice/group.

Therapy with Flortec ameliorates colitis restoring TJ barrier function

To evaluate barrier integrity morphologically, we investigated mRNA levels of TJ occludin and zonula occludens 1 (ZO-1), and their distribution in distal colon. As shown in **figure 7.5**, intensity of staining for occludin (**A**) and ZO-1 (**C**) was significantly diminished in colonic mucosa of DSS mice. This reduction was confirmed by analysis of mRNA levels for these proteins (**Fig 7.5B-D**) (*P<0.05; ***P<0.001 *vs.* CON). In contrast, synbiotic treatment led to substantial preservation of TJ architecture of epithelial cells in DSS colitis, comparable to that observed in control mice. Occludin and ZO-1 remained localized through enterocytes with a continuous staining pattern and also mRNA transcripts were recovered (#p<0.05; p<0.001 *vs.* DSS).

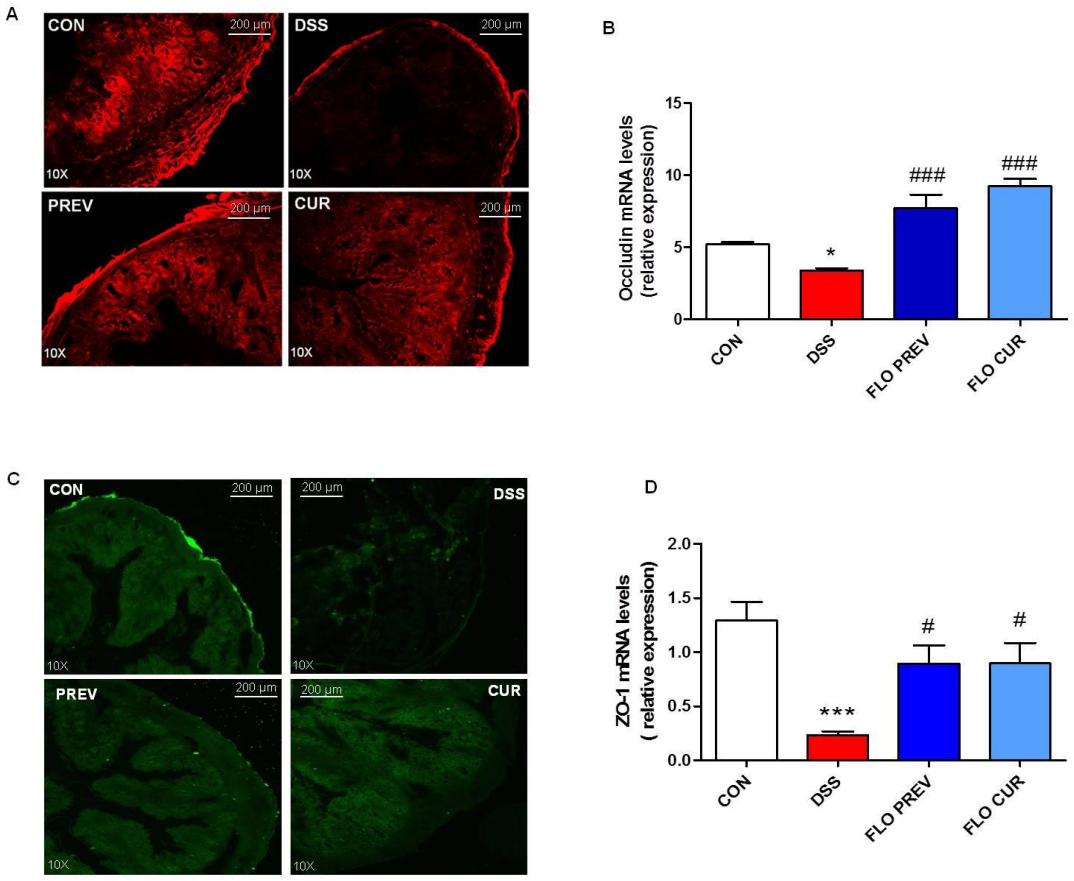


Fig. 7.5. TJ impairment in colon tissue from DSS mice. Representative immunofluorescent images of Occludin (A) and ZO-1 (C) reveals TJ loss in distal colon sections of DSS- challenged mice. Synbiotic treatment recovered distribution and mRNA amount of both Occludin (B) and ZO-1 (D). PCR data are mean ± SEM from 7 to 8 mice/group. Immunofluorecence images are representative of 5 slides for each group (Magnification 100X).

Flortec prevents the inhibition of inflammosome complex pathway in DSS-challenged mice

Among members of the NLRP sub-family, NLRP3 and NLRP6 are the better characterized. They has been shown to trigger IL-1β and IL-18 processing and release in response to a variety of pathogen and endogenous danger signals including monosodium urate crystals (MSU), adenosine triphospate (ATP), silica and asbestos [577,578]. As depicted in **figure 7.6** a downregulation of NLRP3 (**A**) and NLRP6 (**B**) mRNA were observed in colon tissue of DSS mice. In the same way also mRNA levels of Caspase 1 and IL-18 were reduced in these animals (**Fig. 7.6C and D** respectively). So DSS treatment led to a significant reduction of several components of inflammosome complex in colonocytes (**P<0.01; ***P<0.001 *vs.* CON). Both therapeutic schemes with synbiotic (PREV and CUR) were able to prevent the downregulation of these components and to preserve the colonocyte's capability to contrast the tissue damage induced by DSS (#P<0.05; P<0.01 *vs.* DSS).

The role of inflammasome–caspase 1–IL‑18–IL‑18R–MYD88 axis in mediating tissue repair in the intestine is summarized in **figure 7.7**

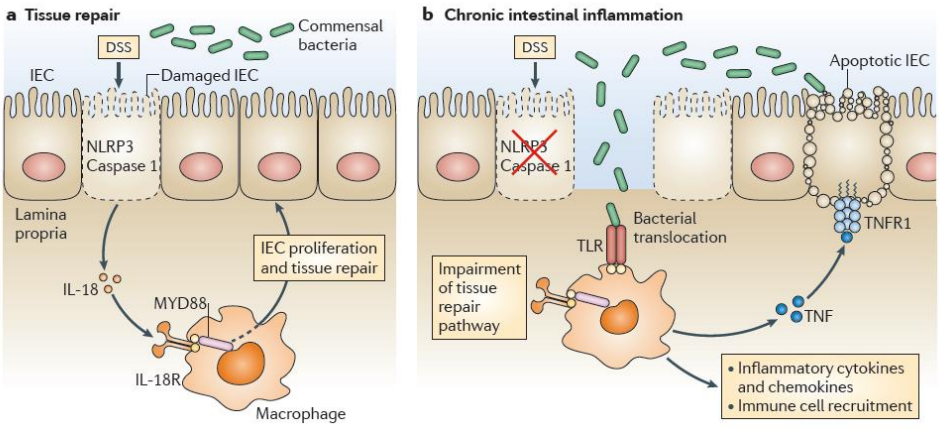


Figure 7.7. The inflammasome–caspase 1–IL‑18–IL‑18R–MYD88 axis mediates tissue repair in the intestine. (a) Following tissue damage with the intestinal epithelial cell (IEC) cytotoxic agent dextran sulphate sodium (DSS), the NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome, which contains NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and caspase 1, assembles in IECs. This leads to the production of interleukin-18 (IL-18), which is then released at the mucosal sites. IL-18 binds the IL-18 receptor (IL-18R), which is expressed by myeloid cells in the lamina propria (and possibly by other cell types) and signals through the adaptor molecule myeloid differentiation primary response protein 88 (MYD88). IL-18 signalling induces compensatory proliferation of IECs and tissue repair. **(b)** If this innate immune signalling pathway is impaired (as observed in mice that are deficient in caspase 1, ASC, NLRP3, IL-18, IL-18R or MYD88), persistent tissue damage leads to the translocation of commensal microorganisms to the submucosa, where they stimulate resident immune cells through Toll-like

receptors (TLRs) and other pattern recognition receptors (not shown). Secretion of cytokines by activated immune cells results in tumour necrosis factor (TNF)-induced IEC apoptosis and chronic intestinal inflammation. TNFR1, TNF receptor 1. *Picture from: Saleh M, Trinchieri G. Innate immune mechanisms of colitis and colitis-associated colorectal cancer. Nat Rev Immunol. 2011 Jan;11(1):9-20. doi: 10.1038/nri2891. Epub 2010 Dec 10. Review.*

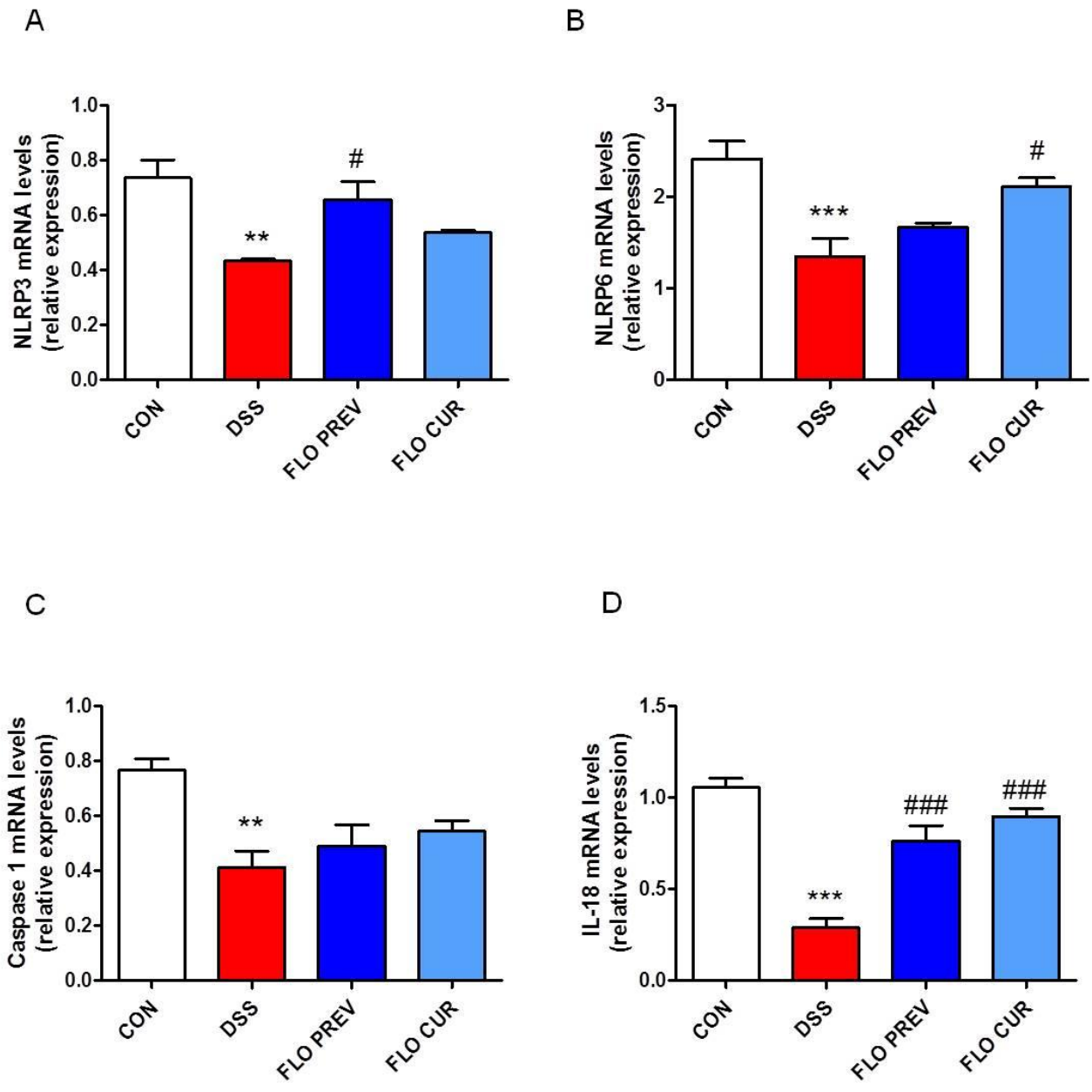


Fig. 7.6. Flortec restores inflammasome components in intestinal epithelium of DSS-challenged mice. (A) mRNA amount of NLRP3 and (B) of NLRP6. (C) and (D) transcriptional levels of caspase 1 and IL-18 respectively. Data are mean ± SD from eight mice/group.

DSS alteres colonic β-defensin expression and decreases mucin production: effect of synbiotic treatment

Given the colonic nature of the injury observed in our model, we sought to assess the expression of select colonic β-defensins in DSS-challenged mice. Significant changes in β-defensin 1 (Defb1) transcript expression were observed in DSS-alone animals (**Fig.7.8A**). Alterations in defensin expression were associated with a reduction in the antimicrobial capability of these mice. In the same way also mucus production was impaired in DSS-challenged mice, in fact, mRNA levels of MUC-1 were significantly reduced compared to control (**Fig. 7.8B**) (***P<0.001 *vs.* CON). In spite of this effects, the curative and in particular the preventive treatments with Flortec, contrasted the downregulation of Defb-1 and MUC-1 induced by DSS in colon tissue exhibiting a protective role on intestinal epithelium (#P<0.05; P<0.01 *vs.* DSS).

Several immune mechanisms work in concert to limit contact between the dense luminal microbial community and the intestinal epithelial cell surface and to maintain homeostasis. Some of them, including defensins and mucin are reported in **figure 7.9**.

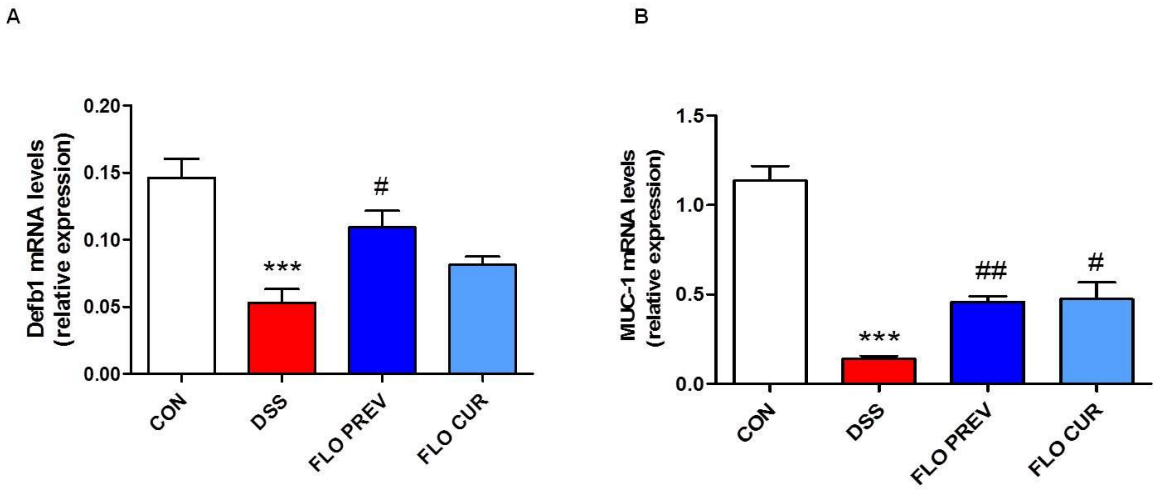


Fig. 7.8. DSS impairs antimicrobial capacity and mucus production in colon tissue. mRNA relative expression of Defb-1 (A) and MUC-1 (B) were recovered by synbiotic treatment. Data are mean ± SD from eight mice/group.

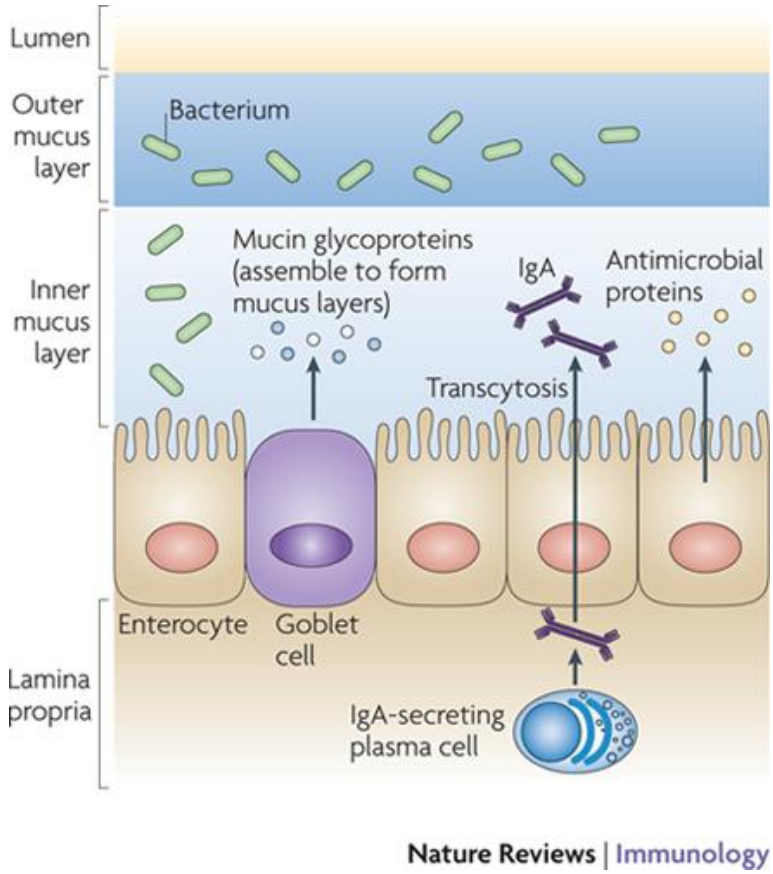


Figure 7.9. Immune adaptations that maintain homeostasis with the intestinal microbiota. Several immune mechanisms work in concert to limit contact between the dense luminal microbial community and the intestinal epithelial cell surface. Goblet cells secrete mucin glycoproteins that assemble into a thick, stratified mucus layer. Bacteria are abundant in the outer mucus layer, whereas the inner layer is resistant to bacterial penetration. Epithelial cells (such as enterocytes, Paneth cells and goblet cells) secrete antimicrobial proteins that further help to eliminate bacteria that penetrate the mucus layer. Plasma cells secrete IgA that is transcytosed across the epithelial cell layer and secreted from the apical surface of epithelial cells, limiting numbers of mucosa-associated bacteria and preventing bacterial penetration of host tissues. *Image from: Lora V. Hooper & Andrew J. Macpherson. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nature Reviews Immunology 10, 159-169 (March 2010) doi:10.1038/nri2710.*

7.4 Discussion

At birth, the gastrointestinal tract is a sterile environment. Initial exposure of the gut to microbes occurs during the birthing process from the maternal fecal and vaginal flora. Within a few months after birth, a relatively stable microbial population is established [3]. This abundant, diverse and dynamic intestinal microflora normally lives in a complex, symbiotic relationship with the eukaryotic cells of the mucosa. About 100 trillion bacterial cells benefit from the constant nutrient flow, stable temperature and 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, inhibit the growth of pathogens, sustain intestinal barrier integrity and maintain mucosal immune homeostasis. Studies from germ-free animals reveal that the absence of resident intestinal microflora results in significant alterations in intestinal structure and function, including slender villi, shallow crypts, low leukocyte count [579], a decrease in the number and density of Peyer's patches [580] and decreased stimulation of migrating motor complexes [581]. In their co-evolution with bacteria, vertebrates develop pattern-recognition receptors, which are activated by specific molecular patterns unique to bacteria, fungi and viruses that are absent in eukaryotes (lipopolysaccharides, peptidoglycan, ssRNA, muramyl dipeptide, flagellins, etc). These include the Toll-like receptors and nucleotide oligomerization domains (NODs). TLRs and NODs are critical for the initiation of innate immune defense responses. Activation of their signaling cascades usually results in the production of pro-inflammatory cytokines. TLR signaling also provides a link between innate and adaptive immunity, as TLR signaling results in the maturation of dendritic cells, which activate adaptive immune responses [114]. Although stimulation of these receptors in most parts of the immune system results in production of inflammatory cytokines, these ligands are not only tolerated by the gut mucosal immune system, but also essential for adaptation to intestinal bacteria and maintenance of homeostasis [582]. The tolerance to the intestinal microflora is not completely understood, but several aspects of commensal physiology have been defined which contribute to their inability to activate the immune system. Some commensal bacteria can modify TLR ligands, resulting in a hypoactive immune response. For example, the endotoxic portion of LPS is pentacylated in many Bacteroides species, and has minimal toxicity [583]. An important feature of commensal bacteria is their inability to penetrate the intestinal epithelial barrier. If some of these organisms do penetrate, they are usually rapidly swallow up by the innate mucosal immune system. Indeed, in healthy host,

the systemic immune system seems to overlook intestinal microflora [584]. Maintaining tolerance to these intestinal bacteria is a remarkable accomplishment achieved by the mucosal immune system, and disturbances in this bacterial-epithelial homeostasis result in considerable deleterious effects on the host. More than 20 animal models of IBD are available [585] and have been widely used to study the efficacy and mechanisms of probiotics in ameliorating inflammation in order to provide support for human clinical trials. In IL-10 knockout mice, *L. plantarum 299v* [586], *L. Reuteri* [587], *L. salivarius subspecies salivarius UCC118* [588] and VSL#3 [279] have all been shown to successfully attenuate intestinal inflammation. *L. GG* prevents recurrent colitis in HLA-B27 transgenic rats after antibiotic treatment, whereas *L. Plantarum* has no effect [589]. Dextran sulphate sodium (DSS)-induced colitis in mice is ameliorated by soluble bacterial antigens extracted from *E. coli (strain Laves)* or by *Bifidobacterium strains Breve, Catenulatum,* and *Longum* [590]. Daily administration of live but not heat-killed auto-aggregating *L. Crispatus* reduces the severity of DSS-colitis in mice [591]. Interestingly, DNA from VSL#3 has been reported to reduce colonic inflammation, thus improving intestinal barrier function in IL-10 KO mice and DSS-induced colitis [171,592]. It is important to note that probiotics should be divided into immunostimulatory and immunomodulatory according to their ability to interact with immune and non-immune cells, and their clinical use should be tailored accordingly. For instance, LGG, which is immunostimulatory has been shown to be more appropriate in the prevention of nosocomial rotavirus-dependent diarrhea in infants [593] or in decreasing the incidence of atopic dermatitis [594], than as an additive therapy in children with Crohn's disease (CD) [595] or in CD patients after surgery [596]. In contrast *L. paracasei,* which is immunomodulatory, may be used to dampen inflammatory responses and may be recommended to maintain the remission phase in IBD. In fact, *L. paracasei* was a poor inducer of cytokines (both inflammatory and non-inflammatory) and impacted on the ability of DCs to produce inflammatory cytokines in response to pathogens, suggesting that this could represent a new class of immunomodulatory probiotics. On the basis of these instances the aim our work was to evaluate the preventive and curative effects of Flortec on a murine model of colitis induced by DSS. Flortec is a synbiotic formulation containing a probiotic component as *Lactobacillus paracasei strain B21060* with a prebiotic component represented by fructo-oligosaccharides and arabinogalactane. DSS at 2.5% (wt/vol) in drinking water was administered *ad libitum* to the animals for 5 days followed by 7 days of washout. After these 12 days the animlas were sacrified. This model is able to reproduce the complexity and the event's cascade which characterize the development of moderately active ulcerative colitis [597]. In our experiment, DSS mice showed a reduction of body weight only after DSS challenge, while both therapeutic schemes

(PREV and CUR) prevented this loss in DSS-challenged mice. Our result appear in contadiction to data in letterature because we didn't observe weight loss immediately after DSS administration. Anyway many factors can contribute to this event: primary the percentage of DSS, secondary the time of exposure to ulcerative agent. Some papers show a strong reduction of weight when mice are exposed to DSS assumption for five or more days and when DSS is used at higher percentage then our one [598].

Feighery et al., [599] showed the effects of *Lactobacillus salivarius 433118* on intestinal inflammation, immunity status and *in vitro* colon function. The authors used DSS 2.5% (wt/vol) for 5 days and then returned to normal drinking water for an additional 7 days. Even in their conditions DSS-challenged mice exhibit weight reduction after DSS treatment but this strain was not able to prevent this reduction. Our results on mice weight change were observed also in others DSS induced colitis protocols [231,600]. The protective effect of Flortec was evident also on colon health. Both treatments reduced colon shortening and ameliorated necrosis and bleeding induced by DSS. About histological analysis, H&E staining of distal colon sections revealed crypt distruction and loss of tissue architecture in DSS mice. Flortec treatment was able not only to preserve mucosa structure and crypt organization, but also to reduce the growth of MALT shown in DSS sections. This effects of synbiotic display its ability both to protect tissue integrity and to modulate immune response in colonic mucosa remarking immunomodulatory skills of this Lactobacillus strain. Our model displays that DSS administration led to a strong exacerbation of disease activity at the end of experimental time while both therapeutic schemes with synbiotic protected DSS-challenged mice from colitis symptoms. On the basis of immunomodulatory skills of *Lactobacillus paracasei B21060*, we searched for immune cells involvement in this model of ulcerative colitis. Our results show that the DSS promotes local epithelial induction of CCL5 transcription leading to an exaggerated autoinflammatory response. CCL5 is chemotactic for T cells, neutrophils, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. This chemokine is potently induced by bacterial and viral infections, and in turn it induces massive recruitment of a variety of innate and adaptive immune cells carrying CCR1, CCR3, CCR4 and CCR5 [601]. Interestingly, both PREV and CUR have been shown to reduce CCL5 transcription in colon tissue. Since neutrophil infiltration and crypt abscess are histological features common to both ulcerative colitis and the murine DSS-induced colitis model, neutrophils may play a critical role in the pathogenesis of both damages. NE is a major secretory product from neutrophils and is capable of hydrolyzing most connective tissue components, leading to tissue injury at inflammatory sites. In this regard we performed Real-Time PCR analysis of NE transcript in colonic mucosa to

confirm CCL5 data about neutrophil recruitment. Inflammed colon sections from DSS mice exhibited significant levels of neutrophil elastase mRNA, confirming so the strength link between this marker of neutrophils and ulcerative colitis. Two important classes of products that are released by activated neutrophils are reactive oxygen intermediates (ROI) and neutrophil-derived proteases [602,603]. MPO assay represent another indicator of immune cells recruitment in inflammed tissue. In accordance with NE data, high levels of U.MPO/mg protein were discovered in colon of mice treated with DSS. Similarly, nitrosylated-proteins levels derived from ROI production, resulted up-regulated in these animals. These data confirme in our experimental protocol of DSS-induced colitis, an evident correlation between immune cells infiltration, particulary neutrophils, and inflammation leading tissue damage. As consequence of oxidative stress and tissue damage, also malondialdehyde (MDA) levels resulted increased in colon tissue of DSS mice. In fact MDA is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation and can be considered as indicator of tissue oxidative stress and damage [604]. In support of a role for probiotics in modulating immunity, rectal administration of *Lactobacillus species* prevented the development of spontaneous colitis in IL-10 gene deficient (IL-10-/-) mice [268]. Similarly, a reduction in mucosal inflammation was detected in IL-10-/- mice in response to long-term oral administration of *Lactobacillus salivarius ssp. salivarius UCC118* (UCC118) in milk, which was also associated with a reduced rate of progression to dysplasia and colonic cancer [269]. Attenuation of inflammation by bifidobacteria and Lactobacillus in IL-10-/- mice may be in part related to induction of a reduced mucosal and systemic T helper (Th)1-type cytokine response [270]. In another example, while pretreatment with *L. casei subsp. Shirota* could not prevent the induction of DSS-induced colitis in mice, it did appear to improve the condition when administered at the time of colitis induction [605]. Our data demonstrated that Flortec was able to down-regulate all markers of immune cells infiltration and so to reduce inflammation and tissue injury. This antinflammatory effect of synbiotic was evident also from analysis of TNF-α, COX-2 and IL-10 transcriptional levels in colonic mucosa. In fact, Flortec reduced significantly pro-inflammatory mediators and restored IL-10 mRNA levels in colon tissue. IL-10 can inhibit the expression of IL-1α, IL-1β, IL-6, IL-12, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, M-CSF, TNF, leukemia inhibitory factor and platelet activating factor produced by activated monocytes or macrophages. It exerts strong immune inhibitory function [266] and plays a major role in the immune tolerance of intestinal mucosa [606,607]. Lack of this cytokine, in fact, in knock out mice is responsible of spontaneous colitis in these animals [608]. Another protective cytokine is adiponectin. This is an adipokine (secreted from adipose tissue), which plays important role not

only in lipid and carbohydrate metabolism but also in anti-inflammatory process [609]. In our model, serum adiponectin levels were dow-regulated by DSS administration while Flortec prevented this action confirming anti-inflammatory property of this synbiotic formulation when used as preventive or curative treatment. Our data display an inflamed condition of colon tissue and a pro-resolving effect of the synbiotic, but this anti-inflammatory ability is a consequence rather than a mechanism of action of *Lactobacillus paracasei*. Beyond its immunomodulatory properties which respresent a specific mechanism of probiotics, these can act with aspecific mechanisms as enhancement of epithelial barrier function, competitive exclusion of bacteria along epithelium and modification of local microenvironment. In another study of our group we demonstrated that Flortec was able to ameliorate intestinal barrier function altered by high fat diet feeding fo 6 weeks in rats [113]. In this experiment, in fact, *Lactobacillus paracaresi B21060* improved gut permeability restoring mRNA levels and tissue distribution of tight junctions proteins ZO-1 and occludin among colon epithelium. Furthermore, the loss of ZO-1 and increased permeability preceded the development of significant intestinal inflammation suggesting that in DSS colitis, alterations in the TJ complex occur before the intestinal inflammation and not as a consequence of it. These changes in the TJ complex may facilitate the development of the inflammatory infiltrate seen in colitis [433]. Since alterations in TJ expression and distribution could be considered the onset of colon inflammation and ulcerative colitis, we analyzed transcriptional levels of ZO-1 and occludin and their localization among epithelium. Our findings show that DSS determinated an impairment of epithelial barrier integrity reducing mRNA levels of these TJ proteins. This data was confirmed by immunofluorecence analysis which showed a strong reduction in intensity staining for both proteins. In contrast the synbiotic preserved colonic mucosa from DSS action improving tight junctions expression and distribution among colonic epithelium.

Recent studies assessing the role of the inflammasome in models of experimental intestinal inflammation have revealed that mice deficient in NLRP3 (NOD-, LRR- and pyrin domain-containing), NLRP6, apoptosis-associated speck-like protein containing a CARD (ASC) or caspase-1 exhibit enhanced susceptibility to DSS and 2,4,6-trinitrobenzenesulfonic acid (TNBS) [610,611]. Zaki et al., [612] reported that NLRP3−/− mice exhibited severe transmural inflammation following oral DSS treatment, a phenotype that was dependent on NLRP3- deficiency in non-bone-marrow-derived tissues. Dupaul-Chicoine et al., [613] also found that loss of NLRP3 resulted in more severe DSS colitis and again it appeared that this was dependent on non-bone marrow derived tissues. They also found that the increase in colitis severity was due to the impaired IL-18 processing and that the phenotype could be partially reversed with exogenous IL-18 [613]. Allen et al., [614] also

found that loss of NLRP3 resulted in increased DSS-induced intestinal injury and inflammation but their chimeric studies found that the increased disease severity was dependent on the loss of NLRP3 in bone-marrow-derived cells. Similarly, in mice deficient in NLRP6, caspase-1, or IL-18, gut microbial ecology is altered, with prominent changes in the representation of members of several bacterial phyla. Strikingly, this altered microbiota is associated with a colitogenic phenotype that is transmissible to cohoused wild-type mice, both early in postnatal life and during adulthood [611]. Futhermore, in these animals aberrant microbiota promotes local epithelial induction of CCL5 transcription as a downstream mechanism, ultimately leading to an exaggerated autoinflammatory response [611]. Normally, following tissue damage with the intestinal epithelial cell (IECs) cytotoxic agent dextran sulphate sodium, the NLRP inflammasome, which contains NLRP3 or 6, ASC and caspase 1, assembles in IECs. This leads to the production of interleukin-18 (IL-18), which is then released at the mucosal sites. IL-18 binds the IL 18 receptor (IL-18R), which is expressed by myeloid cells in the *lamina propria* (and possibly by other cell types) and signals through the adaptor molecule MYD88. IL-18 signalling induces compensatory proliferation of IECs and tissue repair. If this innate immune signalling pathway is impaired (as observed in mice that are deficient in caspase 1, ASC, NLRP, IL-18, IL-18R or MYD88), persistent tissue damage leads to the translocation of commensal microorganisms to the submucosa, where they stimulate resident immune cells through TLRs and other pattern recognition receptors. Secretion of cytokines by activated immune cells results in TNF-induced IEC apoptosis and chronic intestinal inflammation [615]. Therefore, a physiological level of inflammasome activation, triggered by the commensal microbiota in the presence of mucosal injury, is necessary for epithelial cell regeneration and is protective from colitis and colitis-associated colorectal cancer. Here, we showed that synbiotic was able to recover this physiological level of inflammasome activation components impaired by DSS treatment. So beyond the improvement of intestinal permeability, Flortec preserved intestinal epithelial repair functionality in response to injury signals. The mucus layer overlying the epithelium secreted by the goblet cells promotes the elimination of gut contents and provides the first line of defense against physical and chemical injury caused by ingested food, microbes and the microbial products. The major component of the mucus is secreted mucins, large glycoproteins with highly polymeric protein backbone structure, linked to numerous hygroscopic and hydrophilic oligosaccharide side-chains that contribute to the formation of gel-like structure [616]. Commensal bacteria are trapped in the mucus layer, failing to reach the epithelial cell surface, and are eliminated by peristaltic movement [617]. The microbes and microbial products are recognized by the sensor system of the intestinal epithelial cells and the immune cells, activating the host innate

defense system. Balanced and dynamic interactions among mucus layers, intestinal epithelial cells, microbiota, and host immune defense is essential for the maintenance of the intestinal mucosal homeostasis. The disruption in the intestinal homeostasis results in the defective mucus barrier with increased permeability that results in inflammation and injury of the intestinal mucosal cells [618]. Besides, it has been demonstrated that lactobacilli up-regulate the MUC2 and MUC3 mucins and inhibit attachment of enterohemorrhagic *Escherichia coli in vitro* [211], and that a probiotic mixture of lactobacilli and bifidobacteria increase the secretion of mucin, stimulating MUC2 gene expression in the rat colon in vivo [212]. Furthermore, Mack et colleagues showed that some Lactobacillus strain stimulated extracellular MUC 3 secretion following adherence to epithelial cell [285]. Besides, NLR signaling has been linked to the regulation of Paneth cell function and the release of antimicrobial compounds terms defensins [619]. On the basis of these observations, given the colonic nature of the injury observed in our model, we sought to assess the expression of colonic β-defensin 1 and mucin 1 (MUC-1). In our results, marked changes in β-defensin 1 and MUC-1 transcript expression were observed in DSS-treated animals. Conversely, Flortec protected colonic mucosa from this significant reduction. So another possible mechanism of action for this synbiotic formulation is the maintenance of the intestinal mucosal homeostasis regulating mucus and antimicrobial peptides secretion by colonic mucosa.

7.5 Conclusions

In this work we show that this synbiotic formulation is able to enhance intestinal barrier function and epithelial repair capability in response to DSS induced colitis in mice. These effects are able to reduce bacterial traslocation with consequent impairment of immune cells recruitment and reduction of colon inflammation. Besides, probiotics lead to competitive exclusion of negative bacteria along epithelium by stimulating paneth cells to produce antimicrobial compounds and goblet cell to secret mucus, restoring beneficial local microenvironment. On this basis, our data display relevant curative effects of this synbiotic formulation in DSS model of colitis and suggests not only a potential therapeutic role for this agent in this pathology, but also the possibility that a supplement of these lactobacilli might prevent the relapse of ulcerative colitis.

CHAPTER 8: N-(1-CARBAMOYL-2-PHENYL-ETHYL) BUTYRAMIDE, A NEW SYNTHETIC BUTYRATE DERIVATIVE, REDUCES INTESTINAL INFLAMMATION IN DEXTRAN SODIUM SULPHATE-INDUCED COLITIS.

8.1 Introduction

The rate and amount of SCFAs production depends on the species and amounts of microflora present in the colon, the substrate source and gut transit time. SCFAs are readily absorbed after their producion and represent a clear example of the importance of the intestinal ecosystem. SCFAs are organic acids produced by intestinal microbial fermentation of mainly undigested dietary carbohydrates, specifically resistant starches and dietary fiber, but also in a minor part by dietary and endogenous proteins. SCFAs are 2-carbon to 5-carbon weak acids, including acetate (C2), propionate (C3), butyrate (C4), and valerate (C5). SCFAs are essentially produced in the colon. The ratio of SCFA concentrations in the colonic lumen is about 60% acetate, 25% propionate, and 15% butyrate. As a result of increasing concentrations of acidic fermentation products, the luminal pH in the proximal colon is lower. This pH seems to boost the formation of butyrate, as mildly acidic pH values allow butyrate-producing bacteria to compete against Gram-negative carbohydrate-utilizing bacteria, such as Bacteroides spp. [402]. The ability to produce butyrate is widely distributed among the Gram-positive anaerobic bacteria hat inhabit the human colon. Butyrate-producing bacteria represent a functional group, rather than a coherent phylogenetic group. Numerically, two of the most important groups of butyrate producers appear to be *Faecalibacterium prausnitzii*, which belongs to the *Clostridium leptum* (or Clostridial cluster Ⅳ) cluster, and *Eubacterium rectale/Roseburia spp*., which belongs to the *Clostridium coccoides* (or clostridial cluster XIVa) cluster of Firmicute bacteria [403]. Butyrate is the major energy source for colonocytes and is involved in the maintenance of colonic mucosal health [406]. Recently several intestinal and extraintestinal effects of butyrate have been demonstrated [530,620]. Butyrate has been studied for its role in nourishing the colonic mucosa and in the prevention of cancer of the colon, by promoting cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes; inhibiting the enzyme histone deacetylase and decreasing the transformation of primary to secondary bile acids as a result of colonic acidification. Therefore, a greater increase in SCFA production and potentially a

greater delivery of SCFA, specifically butyrate, to the distal colon may result in a protective effect. At intestinal level butyrate exhibits several effects, for example, on transepithelial ion transport, on cell growth and differentiation, on inflammatory and oxidative status, on non-specific intestinal defense mechanisms and finally on visceral perception and intestinal motility [530]. About the mechanism/s of its effects, numerous studies have reported that butyrate metabolism is impaired in intestinal inflamed mucosa of patients with IBD [621]. Recent data show that butyrate deficiency results from the reduction of butyrate uptake by the inflamed mucosa through downregulation of MCT1.

The concomitant induction of the glucose transporter GLUT1 suggests that inflammation could induce a metabolic switch from butyrate to glucose oxidation. Butyrate transport deficiency is expected to have clinical consequences. Particularly, the reduction of the intracellular availability of butyrate in colonocytes may decrease its protective effects against cancer in IBD patients [428]. In spite of these several preclinical and clinical studies analyzing the efficacy of SCFAs mixture or butyrate alone in various models of ulcerose colitis, often the results are contradictory. The equivocal results in clinical studies using enemas may partly be explained by differences in treatment duration, butyrate enemas *vs*. SCFA mixture enemas, differences in concentrations and volumes of these SCFAs and the small number of patients included. Some butyrate-based products are marketed even if their spread is still very limited and greatly understaffed in view of the wide spectrum of possible indications, especially in chronic diseases where it is possible to predict their lasting use. The unpleasant taste and odour make extremely difficult the oral administration of butyrate reducing the compliance. Thus, new formulations of butyrate with a better palatability, which can be easily administered orally, are needed. The purpose of this study is to investigate the efficacy of sodium butyrate (Butyrate) and of its more palatable derivative, the N-(1-carbamoyl-2-phenyl-ethyl) butiramide (FBA), in dextran sulfate sodium (DSS)-induced colitis. We hypothesized that orally administered butyrate compounds, could attenuate colitis and colon injury, with reduction of inflammatory responses *via* gut permeability improvement, suppression of immune cells recruitment, inhibition of HDAC9 activity and restoration of PPAR-γ levels in colon tissue.

8.2 Materials and Methods

DSS-induced colitis and animal treatments

Experimental colitis was induced in ten weeks old BALB/c male mice (25±2 g) (Harlan-Corezzano, Italy) by 2.5% DSS (wt/vol) (MP Biomedicals, Irvine, CA) in sterile drinking water *ad libitum* for five days, followed by drinking water without DSS for twelve days. Mice were randomly divided into six groups (n=10 each group) as following: 1. a control animals (CON), 2. DSS treated mice (DSS), 3. DSS mice treated with sodium butyrate as preventive therapy (PREV Butyrate), 4. DSS mice treated with N-(1-carbamoyl-2-phenylethyl) butyramide (FBA) as preventive therapy (PREV FBA) 5. DSS mice treated with sodium butyrate as curative therapy (CUR Butyrate). 6. DSS mice treated with FBA as curative therapy (CUR FBA). We have recently obtained a high palatable synthetic butyrate derivative, N-(1-carbamoyl-2-phenylethyl) butyramide (FBA; Italian patent RM2008A000214; April 21, 2008). FBA is present in a solid, poorly hygroscopic, easily weighable form, stable to acids and alkalis and capable of releasing butyric acid at small and large bowel level in a constant manner over time. This product has demonstrated a toxicological profile comparable to that of butyrate; it shows physicochemical characteristics distinctly more suitable for extensive clinical use than those of butyrate. A particular aspect of FBA is that it does not present the unpleasant odour of butyrate and is practically tasteless, thus making possible to overcome the main limitation to the use of butyrate in the therapeutic field, namely its very poor palatability. Moreover, the solubility of FBA in water is satisfactory in that it produces clear solutions up to the concentration of 0.1 M and suspensions for higher concentrations. The oral treatment with Butyrate (20 mg/kg/die) or FBA (42.5 mg/kg/die), started 7 days before DSS challenge (PREV) or two days after (CUR) DSS challenge and continued for all experimental period (20 days). DSS group without pharmacological treatments received H_2O+Tween20 0.01% as both drug's vehicle. Colitis was assessed by the daily monitoring of body weight, stool consistency and fecal blood. All procedures involving animals were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 of Ministero della Salute and associated guidelines of the European Communities Council Directive of November 24, 1986 (86/609/ECC). Prior to sample collection, animals, kept overnight fasted, were euthanized by an isoflurane anesthesia, followed by cervical dislocation to minimize pain. All efforts were made to minimize animal suffering. Blood samples from animals were collected by

cardiac puncture and serum obtained. At 20th days mice were killed and full intestine and colons measured. Colon tissue was excised and immediately frozen. Segments of colon were assessed histologically by hematoxylin and eosin staining. In **figure 8.0** the scheme of experimental protocol and animal treatments are summarized.

Figure 8.0. Experimental protocol used in this study

Evaluation of experimental colitis

In all animals, weight, presence of blood and stool consistency were determined daily as previously described [572]. Disease activity index (DAI) was determined by combining scores of a) weight loss b) stool consistency and c) bleeding (divided by 3). Each score was determined as follows, change in weight (0:<1%, 1: 1–5%, 2: 5–10%, 4:>15%), stool blood (0: negative, 2: positive) or gross bleeding (4), and stool consistency (0: normal, 2: loose stools, 4: diarrhea) as previously described [573]. Body weight loss was calculated as the percent difference between the original body weight and the actual body weight on any particular day. Typically in DSS colitis animals will lose 10–15% body weight over the course of 10 days. The appearance of diarrhea is defined as mucus/fecal material adherent to anal fur. The presence or absence of diarrhea was scored as either 1 or 0, respectively, and the cumulative score for diarrhea was calculated by adding the score for each day and dividing by the number of days of exposure. Rectal bleeding was defined as diarrhea containing visible blood/mucus or gross rectal bleeding and scored as described for diarrhea.

Measurement of MPO activity

Proximal colonic tissues were homogenized twice for 30s at 4°C in 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM sodium phosphate, pH 6.0, according to Bradley et al., [575]. The homogenates were clarified by centrifugation at 13000xg for 15 min, at 4°C, and were assessed for MPO activity in 3 ml 50 mM sodium phosphate, pH 6.0, containing 16 mM aqueous guaiacol and 5.9 mM H_2O_2, as previously described [576]. The increase in absorbance was measured for 2 min at 470 nm using The iMark microplate absorbance reader (Bio-Rad). Protein concentrations were determined using the Bio-Rad protein assay. MPO activities were expressed in U.MPO/mg protein with 1 U hydrolyzing 1 μmol H_2O_2/min.

Real-time semi-quantitative PCR

Total RNA isolated from colon was extracted using TRIzol Reagent (Invitrogen Biotechnologies), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas, Ontario, Canada) from 2 μg total RNA. PCRs were performed with Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories). The primer sequences for GPR43, IL-10, IL-6, TNF-α, Occludin and ZO-1 were purchased by Eurofins MWG Operon (Huntsville, AL, USA) and are reported in **Table 8.1**. For Annexin A1 (AnxA1), Ccl2, CD14, CD68, Fpr1, Fpr2, GAPDH, Ly-6G and NOS2 we used QuantiTect® Primer Assays for SYBR Green by Qiagen. The PCR conditions were 15 min at 95°C followed by 40 cycles of three-step PCR denaturation at 94°C for 15 s, annealing at 55 or 60°C for 30 s and extension at 72°C for 30 s. Each sample contained 40-100 ng cDNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/l of each primer in a final volume of 25 μl. The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

Table 8.1. Real-Time PCR Primer Sequence

Target gene	Forward primer (5'→3')	Reverse primer (3'→5')	Accession Number
GPR43	TTCTTACTGGGCTCCCTGCC	TACCAGCGGAAGTTGGATGC	NM_146187
HDAC9	GCGGTCCAGGTTAAAACAGAA	GCCACCTCAAACACTCGCTT	NM_001271386.1
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	NM_010548.2
IL-6	ACAAGTGGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC	NM_031168.1
MCT-1	GAGCGCGCGAAGCTGCATTTGCT	TGCTCCCAGGCCCGCTTTACA	NM_009196.3
PPAR-γ	CTGCTCAAGTATGGTGTCCATGA	ATGAGGACTCCATCTTTATTCA	NM_001127330.1
Occludin	ATGTCCGGCCGATGCTCTCTC	CTTTGGCTGCTCTTGGGTCTGTAT	NM_008756.2
TNF-α	CATCTTCTCAAAACTCGAGTGACAA	TGGGAGTAGATAAGGTACAGCCC	NM_012675.3
ZO-1	ACCCGAAACTGATGCTGTGGATAGA	AAATGGCCGGGCAGAACTTGTGTA	NM_001163574.1

Immunofluorescence analysis of Ly-6G, Annexin A1 and GPR43

Colonic and liver tissue samples for immunofluorescence were embedded in O.C.T. (Pelco Cryo-Z-T, Ted Pella inc, Redding, California), and cryosectioned (10 µm thick). Tissue sections were then fixed in 4% paraformaldehyde for 10 min at room temperature (RT) and washed in TBS with 0.05% TX-100. For immunofluorescence detection of Ly-6G, sections were blocked with 10% FCS in TBS for 30 minutes at RT and then incubated with a monoclonal antibody anti Ly-6G-FITC (BD Biosciences) overnight at 4°C. To examine co-localization of AnxA1 and GPR43 with Ly-6G, rabbit monoclonal anti-AnxA1 antibody [622] and goat polyclonal anti-GPR43 antibody (Santa Cruz Biotechnology, Inc.) were incubated alongside anti Ly-6G-FITC antibody overnight at 4°C. Sections were washed in 1% FCS plus 0.025% TX-100 and incubated with Alexa-Fluor® 546 goat anti-rabbit IgG (for AnxA1) and with Alexa-Fluor® 594 donkey anti-goat IgG (for GPR43) 1 h at RT. After incubation with secondary antibody, sections were washed in TBS, and then incubated with DAPI to visualize nuclei. Slides were mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, California, USA). Fluorescence was visualized on a Olympus BX51 fluorescence microscope (Olympus) equipped with a DS-QiMc monochromatic camera (Nikon) and X-Cite® Series 120Q Xenon lamp. NIS-Elements BR3.1 software (Nikon) was used for all analyses. Merge images were performed with ImageJ® software. Two negative controls were used: slides incubated with or without primary antibody. Images were recorded at identical gain settings,

performed in duplicate in non-serial distant sections, and analyzed in a double-blind manner by two different investigators. Four image fields were taken of each section.

Serum Adiponectin detection

Whole blood taken through cardiac puncture was centrifuaged after 24h at 1500xg at 4°C for 15 min. So serum obtained was stored at -80°C and then used for adiponectin levels detection by Enzyme Linked Immuno Sorbent Assay (ELISA) (Quantikine® Immunoassay, RD & SISTEMS, Minneapolis, MN) following manufacturer's instructions.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by ANOVA test for multiple comparisons followed by Bonferroni's test, using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA). Statistical significance was set at $P<0.05$.

8.3 Results

Effect of Sodium butyrate and FBA on weight change and disease activity index in DSS mice

As shown in **Fig. 8.1A** both pharmacological treatments (preventive and curative protocol) were able to preserve weight loss induced by dextran sulphate sodium challenge (*P<0.05; **P<0.01 *vs.* CON). As reported in AUC graph, among these therapeutical schemes both curative administrations appeared more effective than preventive ones. Assessment of disease activity index (DAI) after 7 days from DSS end, revealed in DSS-challenged mice, a strong increase of colitis gravity (***P<0.001 *vs*. CON). Instead, Butyrate and FBA (PREV and CUR) significantly prevented the development of ulcerative colitis manifestations (**Fig. 8.1B**) (### P<0.001 *Vs*. DSS).

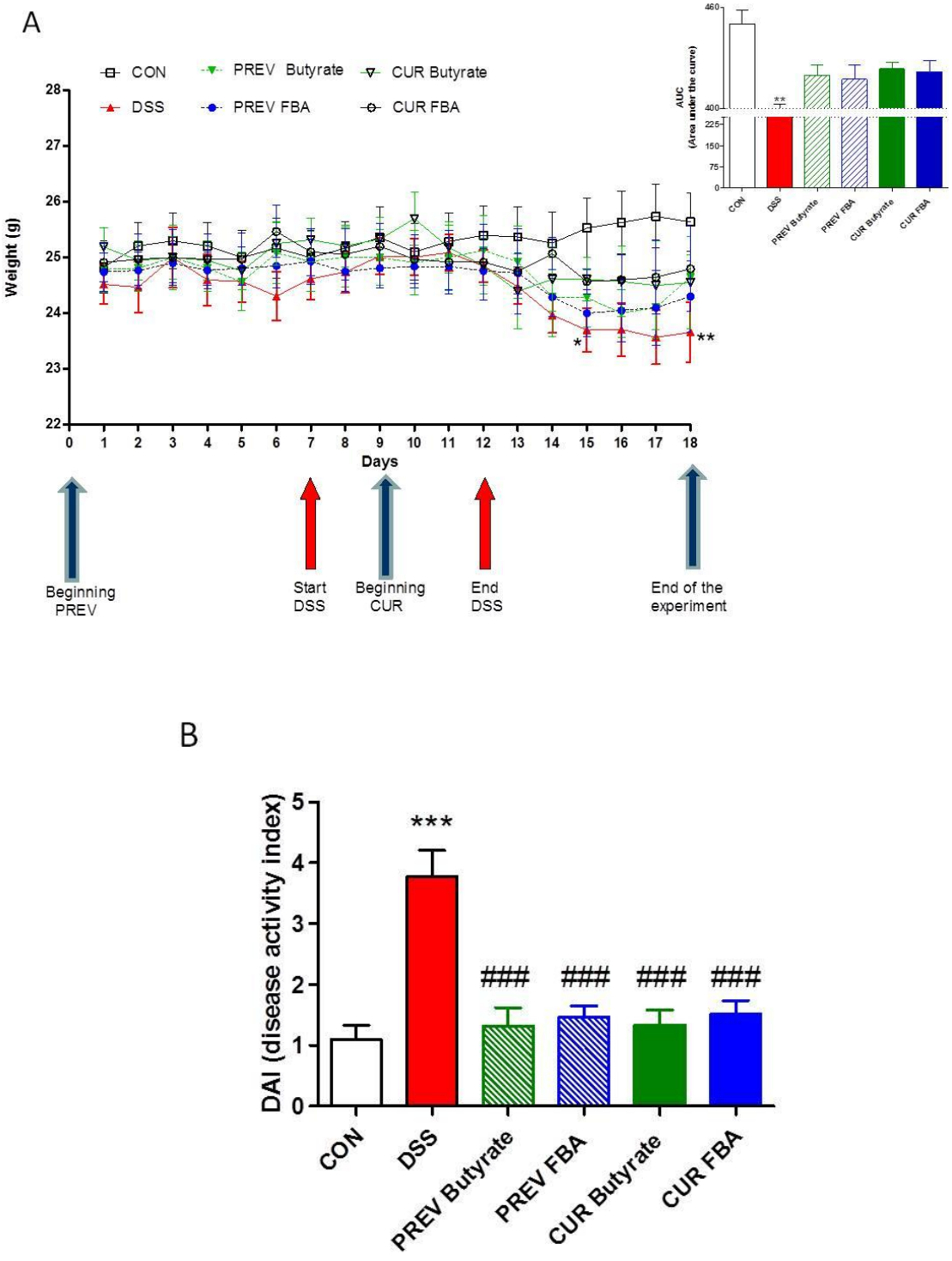


Fig. 8.1 Effects of Butyrate and FBA on induction and recovery from DSS induced colitis.WT mice received 2.5% DSS for 5 days followed by treatment and then returned to normal drinking water for an additional 7. Mice were sacrificed on day 12. (A) Evolution of body weight and (B) DAI values on day 12. Data are mean ± SD from eight mice/group.

Butyrate and FBA improve tissue histopatholgy and prevent colon shortening in DSS- induced colitis

Control mouse colon sections (**Fig. 8.2A**) showed the intact epithelium, well defined crypt length, no edema, no neutrophil infiltration in mucosa and submucosa, and no ulcers or erosions. In contrast, colon tissue from DSS treated mice showed clear and severe inflammatory lesions extensively throughout the mucosa. Ulcers, shortening and loss of crypts were seen focally at the beginning progressing to more extensive areas of mucosal involvement and finally the whole colon. Infiltration of immune cells including neutrophils and lymphocytes were seen in the lamina propria in DSS treated mice. In spite of DSS treatment, Butyrate and FBA were able to protect colonic mucosa structure and to reduce immune cellular recruitment. Besides both preventive and curative treatments ameliorated mucosa integrity and crypt structure improving epithelial surface. Beneficial effects of all therapeutics schemes were shown macroscopically after colon excission. In fact, as depicted in **Fig. 8.2B**, Butyrate and FBA preserved colon from inflammation and bleeding induced by DSS. Futhermore, both therapeutic protocols, in particular curative ones, with Butyrate and FBA reduced colon shortening shown in DSS-challenged mice (**Fig. 8.2C**) (*P<0.05 *vs.* CON; #P<0.05 and ##P<0.01 *vs*. DSS).

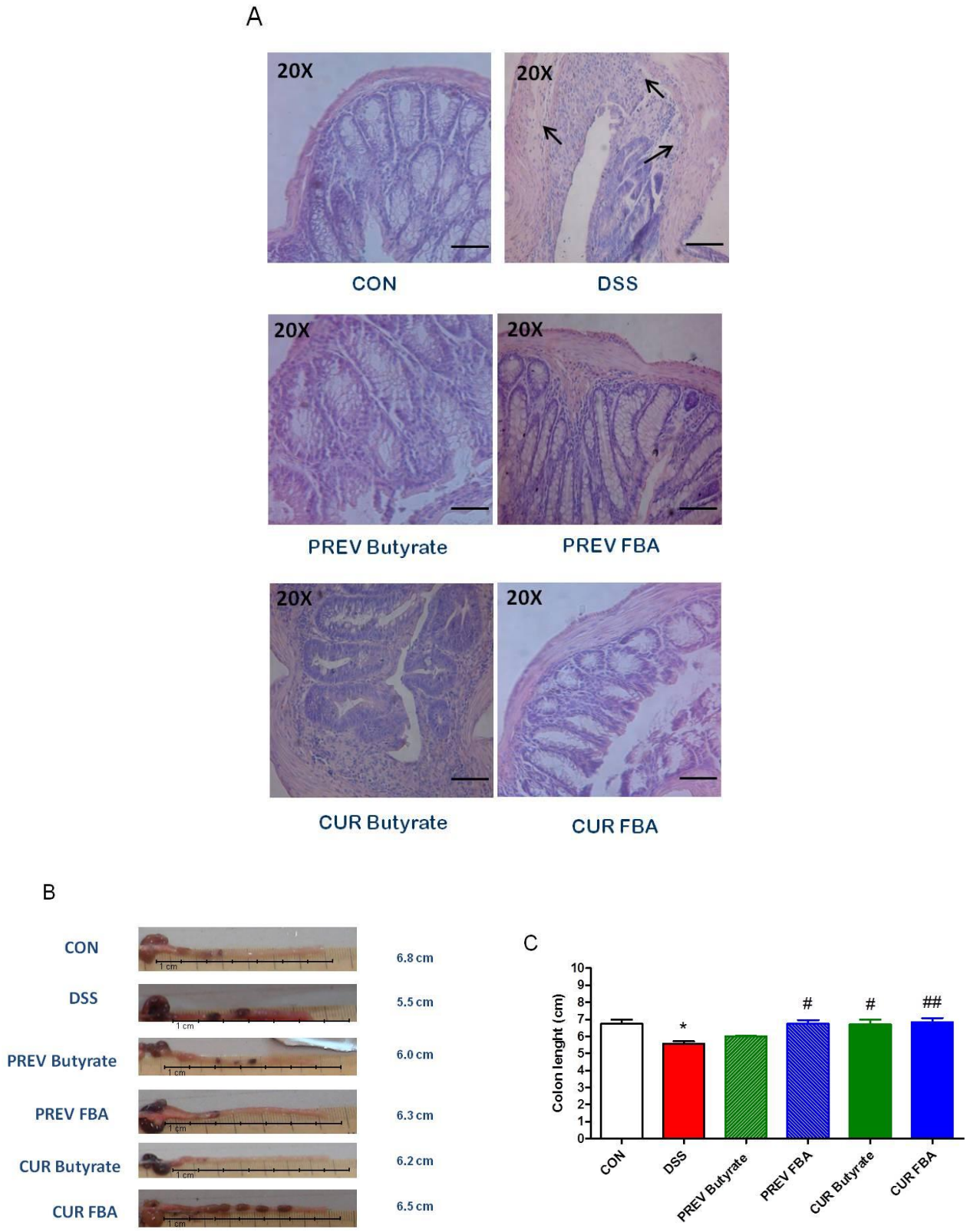


Fig. 8.2. Therapy with Butyrate and FBA ameliorates DSS colitis. (A) Representative histology of the distal DSS colon (H&E staining; original magnification 200X, bar 0.2 mm). Black arrows indicates infiltrated cells in the submucosa. (B) Colon images and (C) assessment of total colon length after DSS treatment. Data are mean ± SD from eight mice/group. Histological images are representative of 5 slides for each group.

Annexin A1 and its receptors are increased in colonic mucosal tissue following DSS treatment:Effect of butyrate formulations

In DSS-challenged mice transcriptional levels of Annexin A1 (AnxA1) and its receptors Fpr1 (Formyl peptide receptor 1) and Fpr2 (Formyl peptide receptor 2), were significantly up-regulated. (**Fig. 8.3A-C**) (*P<0.05; **P<0.01 *vs.* CON). Butyrate and FBA when used as preventive or curative therapy, contrasted with dextran sodium sulphate challenge reducing pro-resolving factor and receptor mRNA levels in colon tissue (#P<0.05; ##P<0.01 *vs.* DSS).

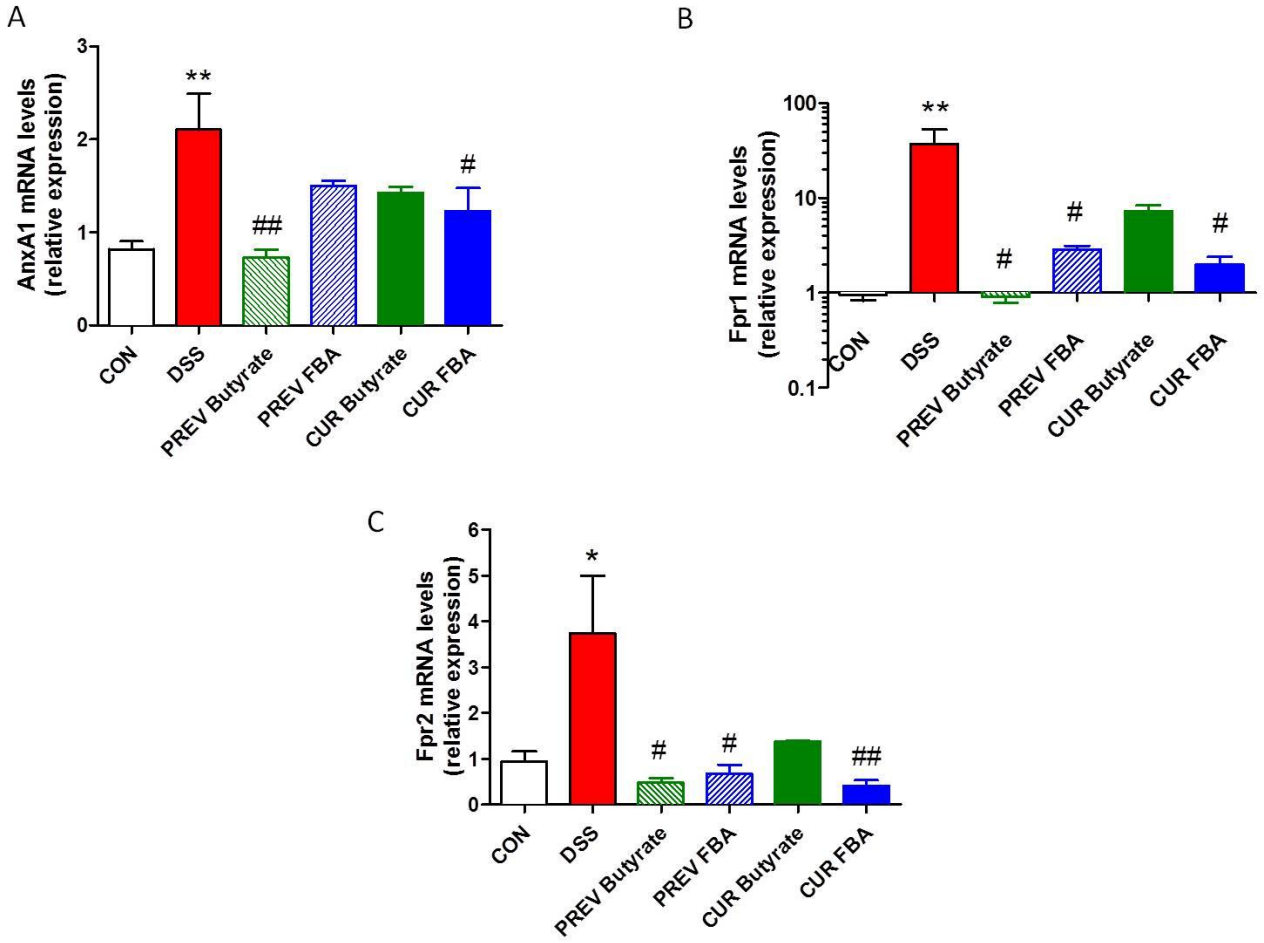
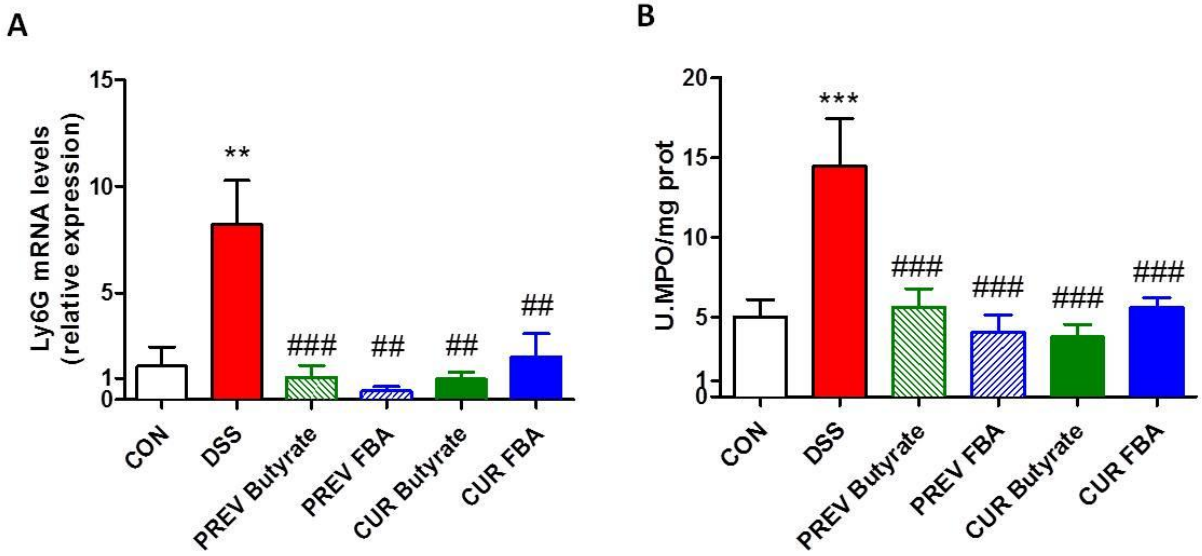


Fig.8.3. Increased expression of AnxA1 and its receptors in colonic mucosal tissues following DSS treatment. Total RNA was extracted from mucosal tissues from control and DSS mice treated or untreated with Butyrate and FBA. Real-Time PCR reveales increased AnxA1 mRNA levels (A). A further diminishment of Fpr1 (B) and Fpr2 (C) mRNA was observed in DSS-challenged animals following treatment with Butyrate and FBA (PREV and CUR). Data are presented as means ± standard error of 5 animals for each group.

Effects of Butyrate and FBA on DSS-induced neutrophil infiltration in colonic mucosa

Given the important role of PMNs in the inflammatory response, we next assessed the infiltration of these cells into the colonic mucosa. PMN infiltration was significantly elevated in colon tissue from mice treated with DSS, as assessed by Ly-6G (a neutrophil granule protease) mRNA levels and MPO activity (**Fig. 8.4A-B**) (**P<0.01; ***P<0.001 *vs.* CON). Furthermore, we used fluorescence microscopy to determine the expression and localization of Ly-6G in colonic mucosal tissue. In control group, a basal low level of this marker expression was observed (**Fig. 8.4C-D**). In contrast, there was a significant increase in Ly-6G staining in colon from DSS-challenged mice. Double-staining experiments revealed high AnxA1 staining in neutrophils, confirmed by a marked co-localization with Ly-6G in colon of mice with active disease (**Fig. 8.4D**). Both therapeutical schemes with Butyrate and FBA were able to reduce neutrophil infiltration. This effect was also evident by impairment of number of PMNs in colonic mucosa (**Fig. 8.4E**) (##P<0.01; ###P<0.001 *vs.* DSS).



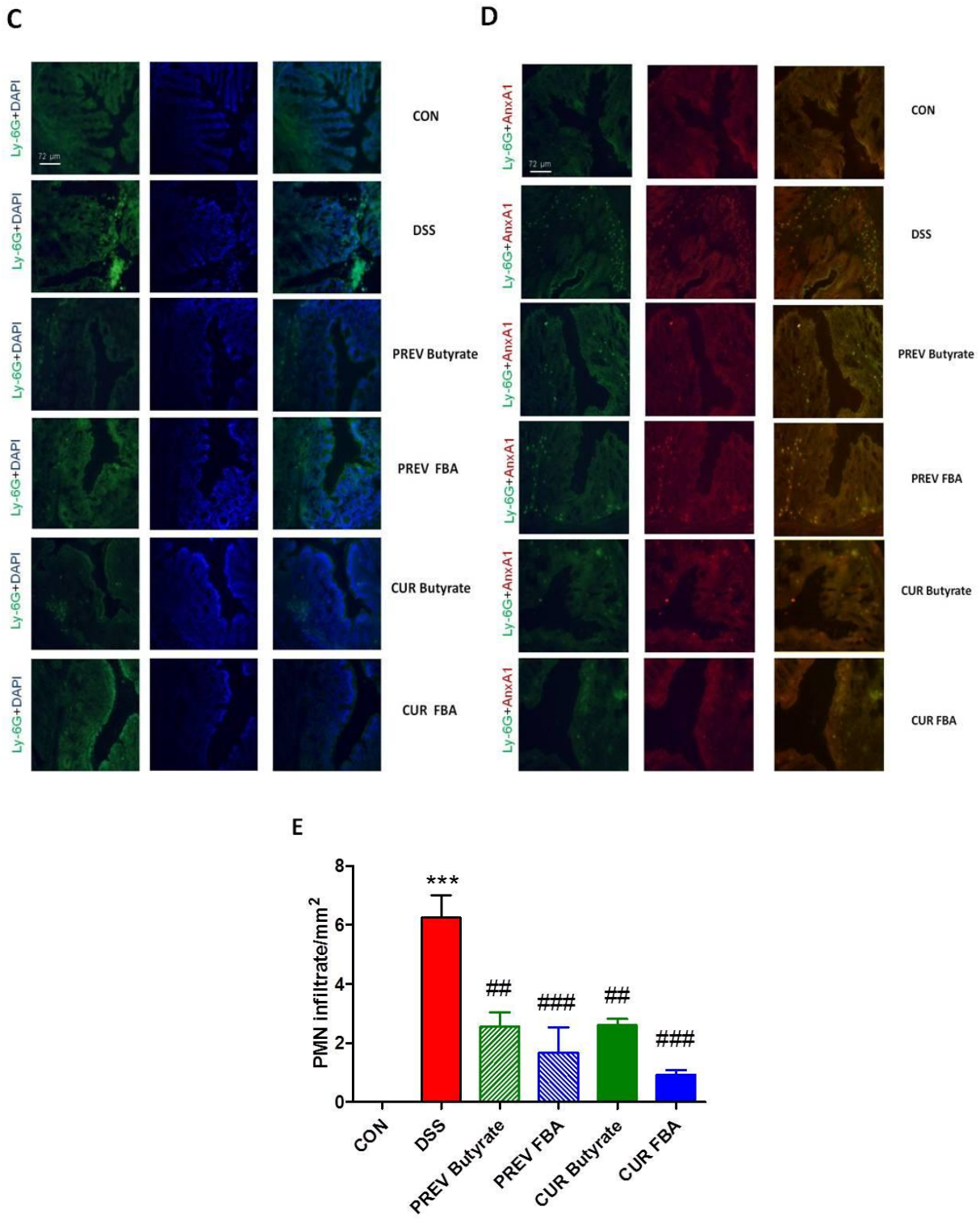
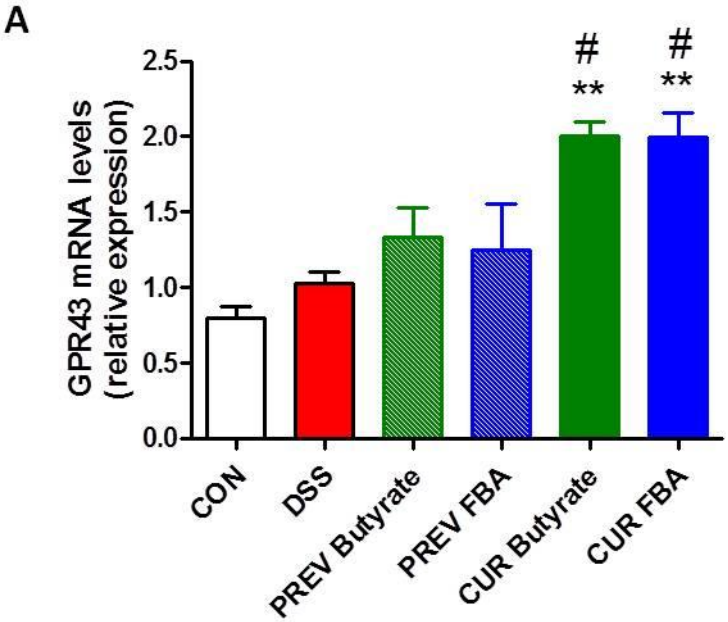


Fig. 8.4 . Butyrate and FBA reduce PMN infiltration in colonic mucosa. (A) Ly-6G transcriptional levels and (B) MPO activity were increased in DSS-challenged mice untreated with Butyrate or FBA. (C-D) Immunofluorescence detection of Ly-6G (green) and AnxA1 (red) demonstrates that PMN infiltrate was increased in DSS-alone group compared to control, Butyrate and FBA groups. Furthermore AnxA1 (red) staining could be localized to infiltrating PMNs (Ly-6G+AnxA1 yellow staining) (C). Real-Time data are presented as means ± standard error of 5 animals for each group. Immunofluorescence staining are representative of 3 slides for each group. Magnification 200X. (E) PMN infiltration score was obtained by counting PMN cellularity in four random mucosal and submucosal views of three different sections from the descendent colon and was expressed as number of cells/ area mm^2.

Butyrate and FBA increase GPR43 expression and distribution along intestinal epithelium in colonic mucosa

As depicted in **Fig. 8.5A** Butyrate and FBA, in particular curative protocol, were able to increase GPR43 trascriptional levels compared to control and DSS-untreated mice (**P<0.01 *vs.* CON and #P<0.05 *vs.* DSS). This effect was also evident by immunofluorecence staining for GPR43 (**Fig. 8.5B**). Furthermore, as shown in **Fig. 8.5C**, this receptor was particularly expressed along intestinal epithelium, and only in DSS-alone group was co-localized with PMNs positive cells. These data suggested involvement of GPR43 in neutrophil recruitment under inflammatory condition and confirmed that Butyrate and FBA reduced PMN infiltrate. Furthermore, to confirm the authenticity and accuracy of GPR43 staining in colon tissue, we performed an immunofluorescence analysis for this receptor also in liver tissue from control mice. We chose liver because in normal conditions it doesn't exprime GPR43. In fact, as depicted in **Fig. 8.5D** we didn't observe differences between liver sections stained with GPR43 and its isotype negative control. So, we confirmed that differences in staining intensity observed among colon sections from our experimental groups, were due to various levels of GPR43 expression and not to background staining.



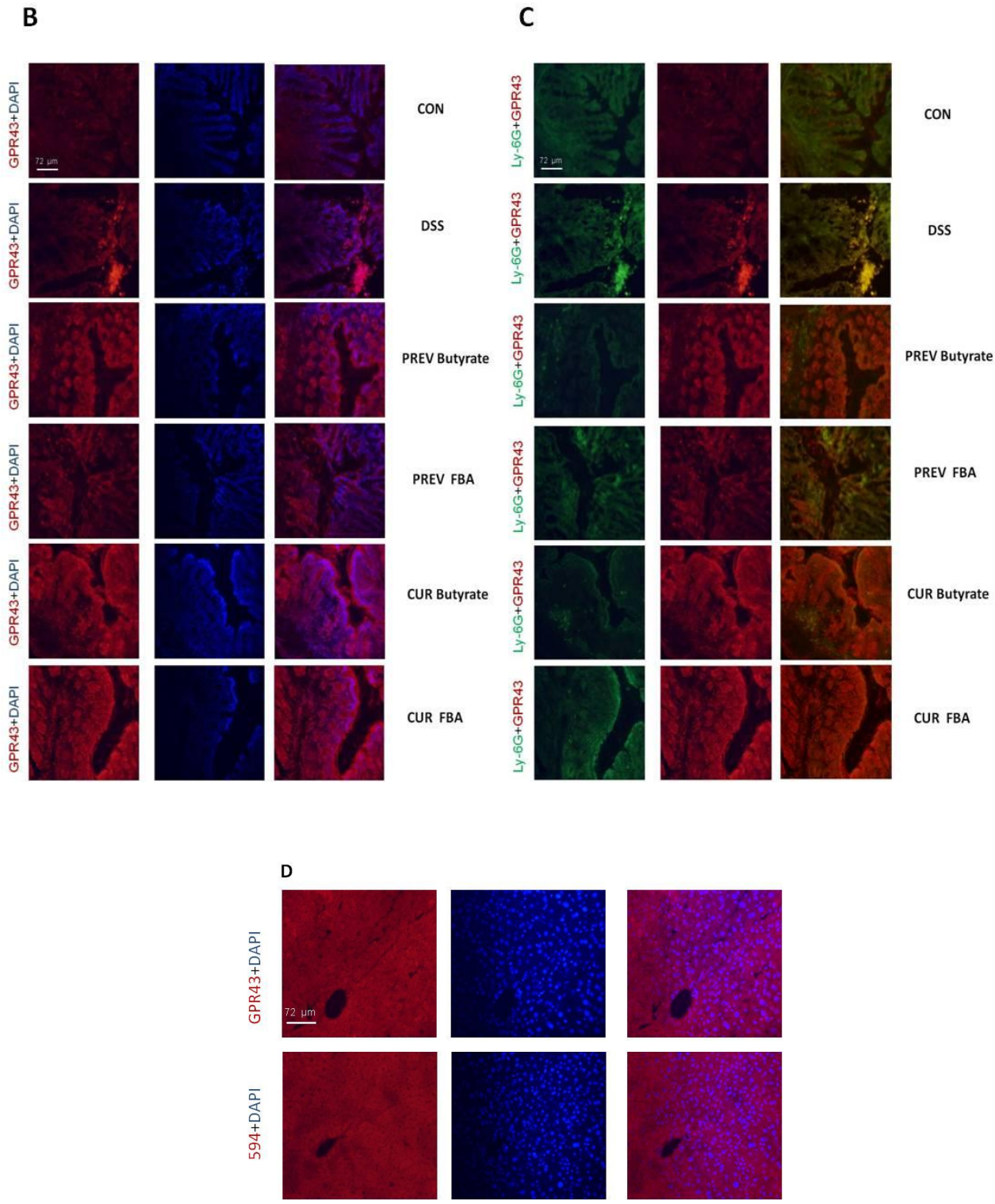


Fig. 8.5 . Effect of Butyrate and FBA on GPR43 expression and localization in colonic mucosa. (A) Real-Time PCR of GPR43 in colon tissue. (B) Immunofluorescence staining of GPR43 (red) and DAPI (blue) on distal colon sections. (C) Double staining of GPR43 (red) and Ly-6G (green) (Ly-6G+GPR43 yellow staining). (D) Immunofluorescence analysis of liver GPR43 content using primary associated with secondary antibody (up) or secondary antibody alone (down). Real-Time data are presented as means ± standard error of 5 animals for each group. Immunofluorescence staining are representative of 3 slides for each group. Magnification 200X.

Effects of butyrate-based compounds on mucosal infiltration by monocytes / macrophages

Butyrate and FBA reduced monocytes infiltration in colonic mucosa by impairment of chemokine expression and lowering macrophages markers. In particular, either pharmacological treatments, both preventive and curative protocol, preserved NOS2, CD14 CD68, Ccl2 enhancement induced by DSS challenge (**Fig. 8.6A-D**) (*P<0.05; **P<0.01 *vs.* CON; #P<0.05; ##P<0.01 *vs.* DSS).

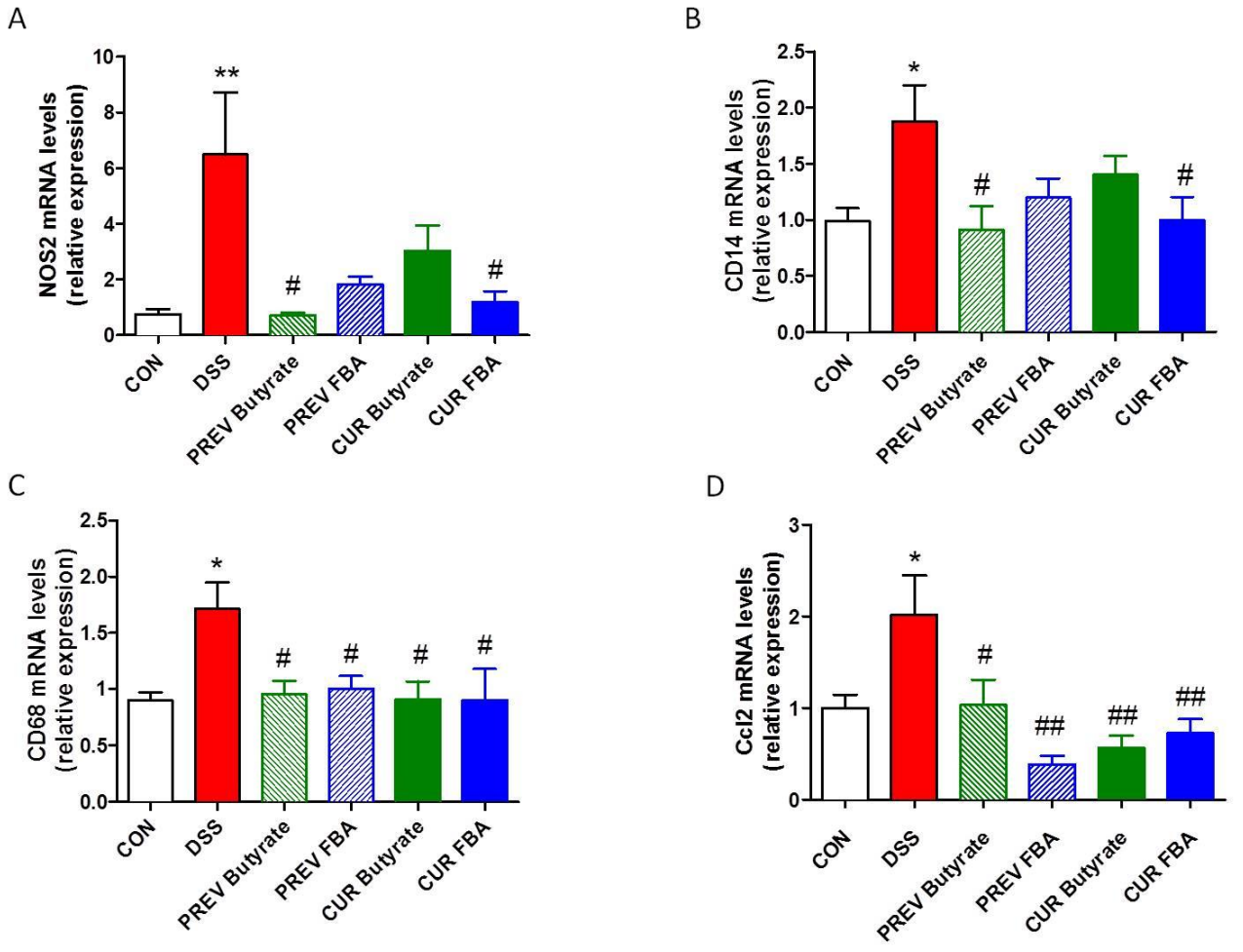


Fig. 8.6. DSS treatment induces up-regulation of monocytes / macrophages markers in colon tissue. Relative amount of (A) NOS2, (B) CD14, (C) CD68 and (D) Ccl2 mRNA was significantly increased in DSS-challenged mice treated with vehicle alone. Both Butyrate and FBA were able to prevent infiltration of immune cells reducing chemoattractant release in colonic mucosa. Data are presented as means ± standard error of 5 animals for each group.

Effect of Butyrate and FBA on inflammatory mediators in colon and serum

Inflammatory state in colonic mucosa was estabilished by TNF-α and IL-6 transcriptional levels evaluation (**Fig. 8.7A-B**). Both these cytokines were significantly up-regulated in DSS-challenged mice, conversely mRNA levels of IL-10, an anti-inflammatory mediator, were reduced (*P<0.05; **P<0.01 *vs.* CON). Protective effect of Butyrate and FBA (PREV and CUR) was shown not only in reducing pro-inflammatory cytokines TNF-α and IL-6 but also in recovered IL-10 and adiponectin, (another anti-inflammatory adipokine), levels in colon tissue and serum respectively (**Fig. 8.7C-D**) (#P<0.05; ##P<0.01; ###P<0.001 *vs.* DSS).

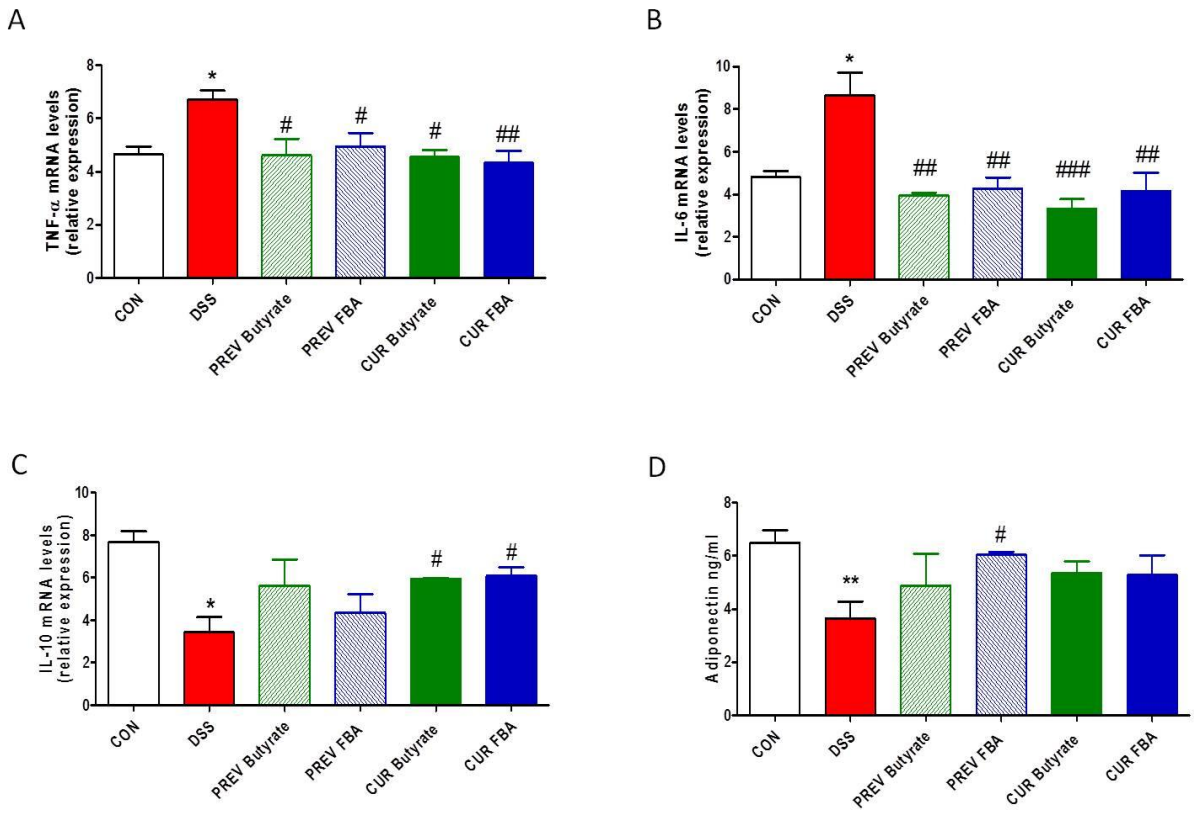


Fig.8.7. Butyrate and FBA inhibit secretion of pro-inflammatory mediators and restore anti-inflammatory cytokines. mRNA trascriptional levels of (A) TNF-α, (B) IL-6 and (C) IL-10 in colon tissue. (D) Serum levels of adiponectin. Data are presented as means ± standard error of 5 animals for each group.

DSS-challenge impairs butyrate uptake and modulates pro-inflammatory response in colonocytes

As depicted in **Figure 8.8A-B**, DSS-challenged mice showed lower mRNA levels of MCT1 and PPAR-γ compared to control group. Furthermore, dextran sodium sulphate was able to induce significantly mRNA transcript of HDAC9 (**Fig. 8.8C**) (*p<0.05; ***P<0.001 *vs.* CON). Instead, Butyrate and FBA, in particularly when used as curative treatments, recovered mRNA expression of MCT1 and PPAR-γ, and at the same time down-regulated HDAC9 mRNA levels (#P<0.05; ##P<0.001 *vs.* DSS).

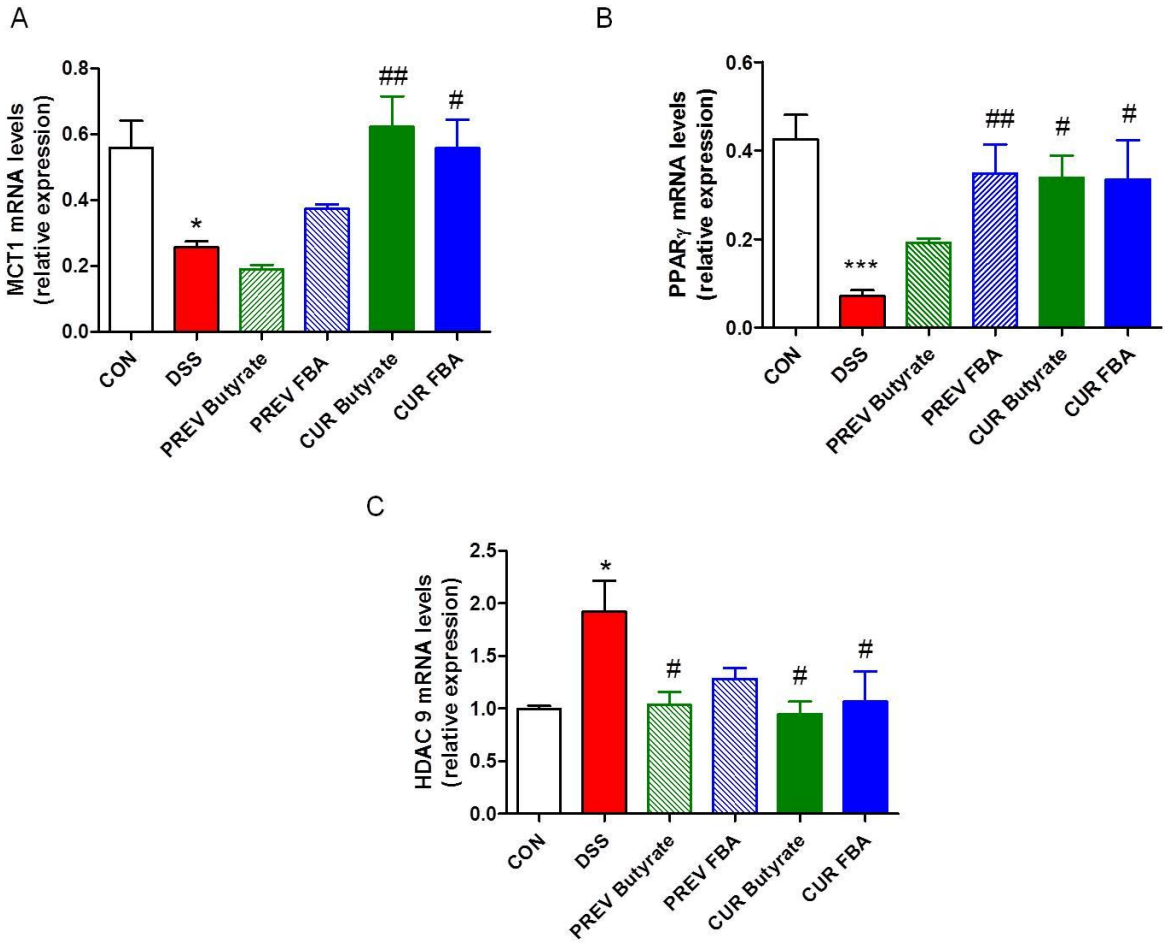


Fig. 8.8. Mechanisms of anti-inflammatory actions adopted by Butyrate and FBA in colonic mucosa. Impaired mRNA expression of (A) MCT-1 and (B) PPAR-γ. Butyrate and FBA act as a histone deacetylase inhibitor reducing HDAC9 trascriptional levels (C). Data are presented as means ± standard error of 5 animals for each group.

8.4 Discussion

In this study we demonstrated that Butyrate and its derived amide FBA are able to protect from colitis injury induced by DSS administration in mice. In particular, they play their effects restoring epithelial barrier integrity and reducing colon inflammation. Effects of butyrate in intestinal disease and its role on colonic functions were evidenced in many studies [530,620]. Since the major limit of butyrate use in clinical practise is its unpleasant taste and odour despite the wide spectrum of possible indications, we obtained a more palatable derivative, the N-(1-carbamoyl-2-phenyl-ethyl) butiramide (FBA), and comparatively evaluated the efficacy of these two compounds in an animal model of colitis induced by DSS. For this purpose, DDS at 2.5% (wt/vol) in drinking water was administered *ad libitum* to the animals for 5 days followed by 7 days of washout. After these 12 days the animals were sacrificed. This model is able to reproduce the complexity and the cascade of events that characterizes the development of moderately active ulcerative colitis [597]. In our experiment, DSS mice showed a reduction of body weight only after five day of DSS administration, while both butyrate-based drugs (PREV and CUR) prevented this loss in DSS-challenged mice. Some papers show a strong reduction of weight when mice are exposed to DSS assumption for five or more days and when they use high percentage of DSS [598]. Anyway many factors can contribute to this discrepancy: primary the percentage of DSS, secondary the time of exposure to ulcerative agent. Our results on mice weight change were comparable to these ones shown in others DSS induced colitis models [231,600]. The clinical and histological changes were determined by phenotypic and pathologic changes such as diarrhea, rectal bleeding, body weight loss and colon shortening, which were the common phenomenon seen in DSS-induced experimental colitis. Diarrhea is due to the increased permeability of intestinal cells or hyper-osmolarity in lumen led by DSS [597]. Weight loss and the shortening of the colon, as indicators for the severity of intestinal inflammation, correlate with the pathologic and histological changes and are consistent markers for colitis. After 5 days of DSS challenge followed by 7 days of washout, Butyrate and FBA, when used as preventive or curative protocol, protected mice from exacerbation of colitis symptoms. IBDs are thought to result from inappropriate and ongoing activation of the mucosal immune system driven by penetration of normal luminal flora due to tight-junction defects of the intestinal epithelial barrier. Our results showed a protective effect of Butyrate and FBA in restoring transcriptional levels of two major tight junction as occludin and ZO-1 (data not shown). These data demonstrate that butyrate has a noticeable effects on colonic epithelial integrity and physiology. The altered TJ structure in ulcerative colitis results in impaired barrier function, which may lead to

increased uptake of luminal antigens and/or adjuvants that overwhelm the net suppressive tone of the mucosal immune system [546]. SCFAs modulate key epithelial cell functions that help to maintain intestinal epithelial barrier integrity against injury [547]; however, the precise innate immune mechanisms of beneficial host-microbial interactions are not yet fully understood. Peng L et al., [623] demonstrated that butyrate enhances the intestinal barrier by facilitating tight junction assembly *via* activation of AMPK in Caco-2 cell monolayer. So, our results showed a protective effect of Butyrate and FBA in restoring transcriptional levels of two major tight junction as occludin and ZO-1. These data demonstrate that butyrate has a noticeable effects on colonic epithelial integrity and physiology. Furthermore, butyrate ability to improve gut permeability was shown in a model of cow's milk allergy (CMA) induced by BLG (β-lactoglobulin) sensitization (Berni Canani et al, *submitted*)*. Here, in fact, mice were treated by daily gavage with 20 mg/kg/day of sodium butyrate, beginning at two weeks prior to sensitization and continuing throughout the sensitization protocol. In this work the authors display that oral administration of sodium butyrate reduced plasma levels of FITC-Dextran and ameliorated gut barrier integrity in mice with CMA. The most important effect of gut permeability integrity is the reduction of bacterial translocation and maintenance of mucosal immunity homeostasis. In fact DAMPs and PAMs penetration determine a strong recruitment of immune cells in infection site and subsequent inflammation establishment. While recent studies have focused mainly on lymphocytes or antigen-presenting cells such as dendritic cells, little is known about the pathogenic role of neutrophils in ulcerative colitis. Indeed, dense neutrophil infiltration and crypt abscess formation are characteristic pathological findings in the inflamed mucosa of these patients [624]. Moreover, in Japan, granulocyte adsorption apheresis therapy has been reported to show a remarkable therapeutic effect in active colitic patients [625]. Taken together these data support the idea that neutrophils can play an important role in the pathogenesis of this pathology. In fact, trans-epithelial migration of PMNs from the microcirculation to the mucosa results in impaired barrier function and destruction of tissue [626]. Consistent with literature data, we observed strong PMN infiltration in colonic tissue obtained from DSS-challenged mice. In fact, in these mice MPO activity (the neutrophil's most abundant enzyme stored in the azurophilic granules), and Ly-6G mRNA levels were significantly up-regulated compared with control group. In our studies, we have used the marker Ly-6G to uniquely identify neutrophils in colonic mucosa. Henderson et al., [627] described that markers such as NIMP-R14 and GR-1, widely used to define the neutrophil lineage, not only detect neutrophils but also subsets of macrophages and even lymphocytes. Our study shows that Ly-6G, reacting only with neutrophils, is a very useful marker to detect specifically cells of the neutrophil

lineage [628], which was also recently described in a study by Tsou et al., [629]. Our results not only show a strong infiltration of neutrophils but also a co-localization between Ly-6G and Annexin A1 positive cells. Annexin A1 is a 37 kDa protein (also known as lipocortin 1; encoded by *ANXA1*) and is a member of a superfamily of annexin proteins that bind acidic phospholipids with high affinity in the presence of Ca^{2+} [630]. It can be considered one downstream mediator of glucocorticoids action. In fact, administration of glucocorticoids to healthy human volunteers leads to an increase in the levels of Annexin A1 expression by circulating monocytes and neutrophils [631]. In resting conditions, human and mouse neutrophils, monocytes and macrophages constitutively contain high levels of Annexin A1 in their cytoplasm [632,633]. Following cell activation (for example, by neutrophil adhesion to endothelial-cell monolayers), Annexin A1 is promptly mobilized to the cell surface and secreted [634]. The molecular mechanisms that are responsible for this rapid secretion are cell specific. Increased expression and secretion of AnxA1 has been reported to occur in inflamed mucosal tissues in rodent models of colitis as well as in human ulcerative colitis [635,636]. Given the homeostatic and anti-inflammatory properties of AnxA1, it is likely that this increased expression might serve to counteract pro-inflammatory and injurious responses in the mucosa. However, experiments demonstrating the functional significance of such increased AnxA1 expression in the injured intestine are lacking. We therefore sought to examine the expression and role of AnxA1 in the DSS-induced acute colitis model. We first examined the transcriptional levels of AnxA1 in colonic mucosa in DSS-treated and untreated BALB/c animals. As shown in **Fig. 8.3A**, real-time PCR analysis of colonic mRNA revealed increased expression of AnxA1 in DSS-treated animals compared with untreated controls. The effects of AnxA1 are mediated via Gi-protein-coupled receptors, Fpr1 and Fpr2 [622]. In similar way, transcriptional levels of both these receptors were increased in DSS-alone mice. It is of interest to note that increased susceptibility, mucosal injury, and clinical morbidity were observed in AnxA1-deficient mice administered DSS. This dys-regulated inflammatory response is compounded by an ablated recovery following withdrawal of DSS administration, thereby providing strong proof-of-concept to the pro-resolving nature of AnxA1 in gut inflammation [598]. Furthermore, Babbin et al., [598] showed an increase of Annexin A1 in both crypt and surface intestinal epithelial cells following 7% DSS treatment for 7 days. In our condition, we found high levels of AnxA1 in colonic mucosa of DSS-challenged mice but this proresolving factor was located principally in neutrophils rather than in epithelial cells. Probably this condition is due to differences in the experimental protocols applied. In fact, 7% DSS administration for 7 days elicits a stronger injury rather than 2.5% administered over 5 days. So, considering AnxA1 involvement in

epithelial repair [637], it could be plausible that upon presence of a severe injury, this pro-resolving factor may be secreted by intestinal epithelial cells. At lower degrees of damage, the mediator is mostly if not exclusively associated with infiltrated neutrophils. In relation to the effects of butyrate on neutrophil chemotaxis, investigations have produced discordant results [109,394,395]. In these cases, butyrate could influence chemotaxis of immune cells but the ultimate effect depended on the type of immune cells and concentration of butyrate applied, as well as on the species [424]. In our study we showed that Butyrate and FBA up-regulated GPR43 mRNA levels in colonic mucosa. At same time we confirmed the involvement of this receptor in neutrophil recruitment. Thus, as shown in **Fig. 8.5C**, GPR43 was co-localized with neutrophil cells only in DSS-challenged mice. So these data suggest that in inflammatory conditions GPR43 can regulate neutrophil chemotaxis while Butyrate and FBA, improving intestinal barrier integrity and reducing immune cells recruitment, are able to increase GPR43 expression only on intestinal enteroendocrine L cells [638]. Taken together these actions, both butyrate-based compounds displayed anti-inflammatory properties.

This protocol for DSS-induced colitis model reproduced the typical events of active and transition phases of self-resolving acute inflammatory process. In this phase, in fact, after penetration of DAMPs and PAMPs due to altered barrier integrity or impaired antimicrobial peptide secretion by Paneth cell and mucus production by Goblet cells, these bacterial products are recognized by resident cells (tissue macrophage, dendritic cell and epithelial cell). Polymorphonuclear leukocytes (mainly neutrophils) are the first cells that extravasate into inflamed tissues followed by mononuclear cells. With progression of the inflammatory response, there is intense leukocyte influx into inflamed tissue. Butyrate and FBA reduced transcriptional levels of Ccl2 gene, which encodes for MCP-1, in colon tissue and thus could impair monocyte recruitment as confirmed reduced CD68 and CD14 mRNAs, both markers of macrophages. Besides, both butyrate-based compounds, as mentioned above, down-regulated AnxA1, Fpr1 and Fpr2 mRNA levels reducing so PMN infiltration. Productive phase of inflammation is characterized by release of pro-inflammatory cytokine and enzymes from immune cells [639]. In our experimental model, establishment of inflammation and tissue injury in DSS-treated mice was confirmed by increase in colon tissue of nitric oxide synthase-2 (NOS2), TNF-α, IL-6 [597] and by reduction in colonic mRNA levels of IL-10 and in serum adiponectin.

IL-10 plays an important role in preventing colitis pathology. In fact, IL-10 knock out are prone to develop spontaneous colitis [608]. Anti-inflammatory effect of preventive and curative treatments with Butyrate and FBA was also evident in reducing pro-inflammatory mediators and in restoring anti-inflammatory ones. In spite of its clinically beneficial effects for patients suffering

from IBD [640], there is a lack of understanding of how SCFAs exert their anti-inflammatory effects. Recent studies of Chang et al. demonstrate that the short chain fatty acid n-butyrate, which is secreted in high amounts by commensal bacterial as *Clostridiales species*, can modulate the function of intestinal macrophages, the most abundant immune cell type in the *lamina propria* [641]. In fact, treatment of macrophages with n-butyrate led to the downregulation of lipopolysaccharide-induced proinflammatory mediators, including nitric oxide, IL-6, and IL-12 but did not affect levels of TNF-α or MCP-1. The authors attribute these effects to inhibition of HDAC activity in macrophages, as it is known that SCFAs inhibit HDAC activity in many cell types [642,643]. Furthermore, several studies demonstrate that inhibition of HDAC9 increases T regulatory cell (T-reg cells) function preventing and ameliorating colitis in mice [644,645]. Butyrate is well known to regulate gene expression epigenetically by inhibiting histone deacetylases (HDACs) [413,642], specifically class IIA and I of the four HDAC classes identified in mammals. Because class IIA HDAC has been reported to suppress T-reg cell expansion [644,646], butyrate may influence histone acetylation of gut CD4+ T cells to regulate epigenetically the transcription of the genes responsible for T-reg cell induction [647]. These findings not only link butyrate to commensal microbe-mediated induction of functional T-reg cells in the colonic mucosa, but also provide molecular insight into the therapeutic application of butyrate and how a metabolite produced by colonic microbial fermentation mediates host–microbial crosstalk for establishment of gut immune homeostasis [648]. Taken together these observations we sought to assess HDAC9 trascriptional levels in colonic mucosa since this enzyme is a member of class IIA of HDAC family. As reported in **Fig. 8.8C**, in DSS-challenged mice we observed a significant up-regulation of HDAC9 mRNA levels while Butyrate and FBA were able to normalize these levels. In fact, butyrate has a role as an anti-inflammatory agent, primarily via inhibition of nuclear factor κB (NF-κB) activation in human colonic epithelial cells [419], which may result from the inhibition of HDAC. NF-κB regulates many cellular genes involved in early immune inflammatory responses, including IL-1β, TNF-α, IL-2, IL-6, IL-8, IL-12, iNOS (or NOS2), COX-2, ICAM-1, VCAM-1, TCR-α, and MHC class Ⅱ molecules [420]. The activity of NF-κB is frequently dysregulated in colon cancer [421] and in IBDs, such as ulcerative colitis and Crohn's disease [422,423]. So, we can suppose that one of possible mechanism of action for its anti-inflammatory effects is due to Butyrate and FBA ability in preventing or reducing NF-κB activation by HDAC9 inhibition. Numerous studies have reported that butyrate metabolism is impaired in intestinal inflamed mucosa of patients with IBD. Recent data show that butyrate deficiency results from the reduction of butyrate uptake by the inflamed mucosa through downregulation of MCT1 [428]. Butyrate transport

deficiency is expected to have clinical consequences. Particularly, the reduction of the intracellular availability of butyrate in colonocytes may decrease its protective effects toward cancer in IBD patients [649]. So in our model we observed a strong reduction of this transporter in colonic mucosa of DSS mice confirming an impairment of butyrate utilization, and oxidative stress, in colonocytes. Both Butyrate and FBA, only when applied alongside a curative protocol, prevented this down-regulation bringing back MCT1 transcriptional levels to physiology. Aside from inhibition of NF-kB activation, butyrate may exert an anti-inflammatory activity through the upregulation of PPAR-γ [650,651]. This nuclear receptor is a ligand-activated transcription factor highly expressed in colonic epithelial cells and its activation exerts anti-inflammatory effects [652]. Genetic ablation of PPAR-γ resulted in increased susceptibility to experimental colitis in rodents [652]. Furthermore, PPAR-γ protein expression is 60% lower in the inflamed colonic mucosa of UC patients compared with that in controls [152]. Modulation of PPAR-γ protein expression in UC may prove to be an interesting treatment for UC. Recent studies have shown that the nuclear receptor PPAR-γ can inhibit NF-kB activation and cytokine expression in monocytes [653] and in murine and human colonic epithelial cells [654,655]. Our hypothesis is that butyrate up-regulates PPAR-γ in epithelial cells, where this acts in a negative feedback loop, uncoupling NF-kB –dependent target genes that are important for inflammatory responses [656]. In fact, Butyrate and FBA restored PPAR-γ mRNA levels in colonic mucosa reducing cytokines release and inflammatory status, and improving intestinal homeostasis.

8.5 Conclusions

In conclusions these data show that Butyrate and FBA are able to improve gut permeability avoiding bacterial translocation and impairing immune cell recruitment. These anti-inflammatory effects are visible as reduction in neutrophil infiltration and reduced HDAC9 transcription in colonic mucosa. These events improve intestinal immune homeostasis and tolerance *vs.* commensal bacteria. In addition, butyrate-based compounds restored colonocyte ability to up-take butyrate after fiber digestion: this occurred with unaltered PPAR-γ levels but blocking NF-kB activation, all leading to colonocyte protection fom the inflammatory condition. Efficacy of Butyrate to limit early molecular events underlying inflammatory process linked to intestinal damage, suggests its potential clinical utility as a preventive and therapeutic strategy for UC. Since FBA does not have the characteristic odor of rancid cheese, this derivative may represent a viable therapeutic alternative to Butyrate, favoring a better compliance and a greater effectiveness.

GENERAL CONCLUSIONS

The study of intestinal microbiota composition and its role in different pathological conditions has greatly helped our understanding on the potential use of probiotics in liver (from simple steatosis to steatohepatitis), and in gut disease. It is now clear that not all probiotics may have the same effects. High-quality preclinical studies and few randomized controlled trials support the therapeutic use of probiotics in gastrointestinal diseases. Unfortunately, these data could not be extrapolated for all probiotic compounds now available on the market. The 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 and ulcerative colitis therapy of probiotics alone or in appropriate synergistic combination between strains or with some prebiotics. Keeping in mind "*primum non nocere*," in the future, nutrients containing pre-probiotics will very likely be considered a new nutritional approach in these patients.

Short-chain fatty acids are important end-products of probiotic fermentation and so they should be considered as "**postbiotics**". Among the SCFAs produced in the human intestine, butyrate has been widely studied and has been shown to play an important role in the maintenance of colonic health. Increased butyrate production in the large intestine seems to be responsible for at least some of the protective effects of fermentable dietary fibre. However, it should be taken into account that the effects of increased butyrate production may be connected with other effects of dietary fibres and their fermentation, such as changes in the composition of the intestinal microbiota and increased faecal bulking. The effects of butyrate are diverse and complex and involve several distinct mechanisms that go beyond the classical impact as an energy source for the intestinal epithelial cells. Frequently described are the effect on gene expression because of the inhibition of histone deacetylase and the suppression of NF-kB activation. Hence, butyrate exerts multiple effects such as the inhibition of colonic carcinogenesis, inflammation and oxidative stress, the improvement of the colonic defence barrier function and the promotion of satiety. These effects are not only at intestinal but also at extraintestinal level. In fact butyrate is able to prevent the main events which underline NAFLD pathogenesis like liver inflammation, insulin-resistance and tissue adipose disfunction.

In the last decade, several new insights into possible mechanisms and effects revealed that butyrate is a pivotal metabolite produced within the large intestine. However, these new insights are

mainly based on *in vitro* data, animal models and some clinical intervention studies. More emphasis should be placed on clinical studies to elucidate the role of butyrate in health and disease.

In these three years our aim was to confirm probiotics and postbiotic efficacy in gastrointestinal diseases. Considering the strength anatomical correlation between liver and gut, we wanted to observe if microorganisms present in gut like probiotics and their postbiotic derivate (SCFAs), were able to prevent or cure not only local intestinal disease but also to limit systemic pathologies like liver steatosis and insulin-resistance. Our results show a protective and curative effect of probiotics and SCFAs in limiting the onset and the progression of these gastrointestinal tract affections. We propose some mechanisms of action for these effects but other studies are necessary to clarify the exact mechanism/s by which probiotics and postbiotics could mediate their efficacy. Surely the first step is represented by restoring gut microflora environment. In fact, probiotics can compete with pathogen bacteria avoiding them to attach intestinal epithelium and so to stimulate immune response. Probiotics can act also indirectly by enhancing postbiotics production which in turn, lowering pH create a unfavorable environment for bacteria overgrowth. Furthermore, both probiotics and SCFAs are able to influence directly colonic functions improving innate immune defense mechanisms. In fact, from our data it appears evident that *Lactobacillus paracasei B21060* and butyrate-based compounds improved not only tight-junction structure, reducing so intestinal permeability but also mucin and defensine production stmulating in this way antimicrobial properties of intestinal epithelial cells. Alteration of tight-junctions or mucin production together with changes in microbiota composition, can be considered as predisposing factors to development of gastrointestinal disease. Specially in western life style, bad alimentary habits taken together with smoke or alchol consumption, represent a favourable situation for the onset of these conditions. In this context, daily assumption of probiotics or directly of their derivate compounds as butyrate, could be considered a valid approach for prevention or treatment of gastrointestinal pathologies. For example, in our model of NAFLD and UC both Flortec and butyrate prevented the beginning of the principal events which underlie progression of these conditions such as insulin-resistance (for NAFLD) and weight loss (for UC).

Many literature data propose one or more possible mechanisms of action for these agents. The question is how probiotics or small molecules as short chain fatty acids, can mediate systemic effects like insulin-resistance, obesity or liver steatosis. We suggest a mechanism based on restoration of intestinal “good” microflora and gut barrier function, avoiding bacterial traslocation which, *via* portal vein circulation, could join liver and here induce inflammation preceding steatosis and IR. In fact in our experimental condition we discovered that treatment with probiotic and

SCFAs was able to restore TLRs pathway in liver tissue. These receptors link bacterial derived products and resulted incresead by high fat diet assumption. How is it possible that PAMPs or DAMPs which normally induce TLRs activation in gut epithelium, carry out their actions also in another tissue? And especially, how is it realizable that probiotics and short chain fatty acids which are produced in gastrointestanl tract, are able to influence metabolic process in liver and adipose tissue? Our results confirmed that modulation of microbiota can improve functionality of other tissues and contribute to host health. Besides, beneficial effects of pro- and postbiotic are principally local. In-fact during inflammatory conditions, due to ulcerogenic agents or intestinal dysbiosis, these compounds restore altered microflora and reduce inflammatory mediators production. To this regard, in our experimendal protocol of sodium dextran sulphate induced colitis, we observed not only preventive but also curative proprieties of Flortec and both butyrate compounds. In fact, our pharmacological treatments exhibited a strong efficacy in reduce colonic inflammation, immune cells recruitment and tissue damage. Furthermore about macroscopic aspects, animals treated with pro- and postbiotics were protected by colitis symptoms such as weight loss, rectal bleeding and diarrhea. These data suggest a potential utility of these compounds not only during acute phase of pathology but also in preventing relapse of disease. About the pathogenesis of colitis, many experimental works underlie the presence of a deregulated microflora and an abnormal immune response against it. In addition to this issue, genetic mutations in TLRs or NOD receptors pathway should play an important role in the onset of this condition. It is well known that *Lactobacillus paracasei B21060* has immunomodulatory abilities and that butyrate is able to influence T-reg activity in colonic *lamina propria*, so it is possible the use of these agents as adjuvant therapy for treatment of mild to moderate colitis. In fact our results confirme an useful clinical application for Flortec, sodium butyrate and FBA as curative or preventive protocol.

Regarding obesity is well known that high fat diet is responsable for establishment of an “high fat microbiota”. This altered microflora extracts from dietary assumption an excessive quantity of calories and this issue could aggravate obesity condition. So maintenance of a good microflora homeostasis, associated with correct dietary habits, contribute to body healthy condition. Nowadays is too difficult specially among paediatric population to obtain respect of healthy dietary habits and correct lifestyle. Furthermore actual therapeutic strategies for treatment of NAFLD and UC are represented by TZDs or metformin and salicylate or corticosteroids, respectively. All these drugs are linked to many collateral effects and could be considerate inappropriate for paediatric patients. So probiotics and butyrate represent a valid therapeutic possibility alone or in combination with traditional drugs for prevention or treatment of NAFLD and mild to moderate ulcerative

colitis. In fact, our data show that Flortec, sodium butyrate and FBA were able to prevent and limit the onset and the progression of NAFLD and ulcerative colitis, improving hepatic steatosis together with insulin-resistance and liver damage, and reducing colonic inflammation and intestinal injury. Furthermore, we obtained a new chemical derivate (FBA) of sodium butyrate which lose bad odour of rancid cheese, and so it could represent a good therapeutical alternative to butyrate, showing a more favorable compliance an a better efficacy in intestinal and extraintestinal diseases.

ABBREVIATIONS:

4-HNE, 4-Hydroxynonenal
4-PBA, Sodium phenylbutyrate 4
5-ASA, 5-Aminosalicylic acid
5-HT, 5-Hydroxytryptamine
AhR, Aryl hydrocarbon receptor
ALT, Alanine transaminase
AMPK, Adenosin monophosphate kinase
AnxA1, Annexin A1
AP1, Activator protein 1
ASC, Apoptosis-associated speck-like protein
AST, Aspartate aminotransferase
ATG16L1, Autophagy-related protein 16-1
BDNF, Brain-derived neurotrophic factor
BLG, β-lactoglobulin
BLIS, Bacteriocin-like inhibitory substances
BrdU, Bromo-2'-deoxyuridine
c-Cbl, Casitas B-lineage Lymphoma
CD, Crohn's disease
CD14, Cluster of differentiation 14
CDH1, Cadherin-1
CFTR, Cystic fibrosis transmembrane conductance regulator
ChAT, Choline acetyltransferase
ChREBP, Carbohydrate response element-binding protein
CLD, Congenital Chloride Diarrhea
CMA, Cow's milk allergy
COX-2, Cyclooxygenase-2
CREB, Phospho-cAMP response element-binding protein
CRP, C-reactive protein
DAI, Disease activity index
DAMPs, Damage-associated molecular patterns
DAP, Diaminopimelic acid
DCs, Dendritic cells
Defb1, β-defensin 1
DGAT1/2, Diacylglycerol acyltransferase 1 and 2
DIO, Diet inducing obesity
DNL, de novo hepatic lipogenesis
DSS, Sodium dextran sulphate
ENS, Enteric nervous system
ERK, Extracellular receptor kinase
FBA, Phenylalanine-butyramide
FDA, Food and Drug Administration
FFAR, Free fatty acid receptor
FFAs, Free fatty acids
FGF21, Fibroblast growth factor
Fiaf, Fasting-induced adipocyte factor
FOS, Fructooligosaccharides
Fpr1, Formyl peptide receptor 1
Fpr2, Formyl peptide receptor 2
GAPDH, Glyceraldehyde-3-phosphate dehydrogenase
GBF, Germinated barley foodstuff
GLP-1, Glucagon-like peptide 1
GLUT4, glucose transporter 4
GM-CSF, Granulocyte-macrophage colony-stimulating factor
GOSs, Galactooligosaccharides

Gpr41 and Gpr43, G protein–coupled receptors 41 and 43
GSTs, glutathione-S-transferases
HbF, Fetal hemoglobin
HCV, Hepatitis C virus
HDAC, Histone deacetylase
HFD, High-fat diet
HLA-DR, MHC class II cell surface receptor
HMGB1, High mobility group box 1
HNF4A, Hepatocyte nuclear factor 4
HOMA, Homeostasis model assessment
HSPs, Heat shock proteins
IBD, Inflammatory bowel disease
IBS, Irritable bowel syndrome
ICAM-1, Intercellular adhesion molecule-1
IECs, Intestinal epithelial cells
IFN-γ, Interferon gamma
IKK β, Nuclear factor-kB kinase-β
IL-18R, Interleukin 18 receptor
IL-6, Interleukin-6
iNOS, Inducible nitric oxide synthase
IPAA, Ileal pouch–anal anastomosis
IR, Insulin resistance
IRF5, Interferon regulatory factor 5
IRGM, Immunity-related GTPase family M protein
IRS-1, Insulin receptor substrate
ITF or TFF3, Intestinal trefoil factors
JAK2, Janus kinase 2
JNK1, c-Jun N-terminal kinases
LAB, Lactic acid bacteria
LAMB1, Laminin beta 1
LCPT1, Carnitine palmitoyl transferase I
LDL-C, Low density lipoprotein- cholesterol
LPL, Lipoprotein lipase inhibitor
LPS, Lipopolysaccharide
MALT, Mucosa-associated lymphoid tissue
MAMPs, Microbe-associated molecular patterns
MAPK, Mitogen-activated protein kinase
MCD, Methionine choline-deficient
MCP-1, Monocyte chemoattractant protein 1
MCT1, Monocarboxylate transporter isoform 1
MDA, Malonyl dialdehyde
MMPs, Metalloproteinases
MSU, Monosodium urate crystals
MUC2, Mucin-2
MyD88, Myeloid differentiation primary response 88
NAFLD, Non-alcoholic fatty liver disease
NASH, Nonalcoholic steatohepatitis
NE, Neutrophil Elastase
NEFA, Nonesterified fatty acids
NF-kB, Nuclear factor-kB
NKT, Natural killer T cell
NKX2-3, NK2 homeobox 3
NLRP, NOD-LRR- and pyrin domain-containing
NLRs, Nucleotide binding oligomerization-domain protein-like receptors
nNOS, Neuronal nitric oxide synthase
NOD2, Nucleotide-binding oligomerization domain-containing protein 2
NOS2, Nitric oxide synthase-2
NRP-1, Neuropilin-1

p38 MAPK, p38 mitogen-activated protein kinase
PAMPs, pathogen-associated molecular patterns
pANCA, Perinuclear antineutrophilic cytoplasmic antibodies
PGC-1α, Peroxisome proliferator-activated receptor (PPAR) coactivator-1α
PKA, Protein kinase A
PLC, phospholipase C
PPAR-□, peroxisome proliferator-activated receptors □
PPAR-□, □peroxisome proliferator-activated receptor □
PRDM1, PR domain containing 1
PTP1B, Tyrosine phosphatase 1B
PTPN2, Tyrosine-protein phosphatase non-receptor type 2
PYY, Peptide YY
RegIIIγ, Regenerating islet derived 3-gamma
RIP, Receptor interacting protein
ROI, Reactive oxygen intermediates
ROS, Reactive oxygen species
SCFAs, Short chain fatty acids
Sirt1, Sirtuin 1
SLC26A3, Solute-linked carrier family 26-member A3
S-NO, S-nitrosothiols
SOCS3, Suppressor of cytokine signalling 3
SREBP, Sterol regulatory element- binding protein
STAS, Anti-sigma factor antagonist
STAT, Signal transducer and activator of transcription
T2DM, Type 2 diabetes
TCDD, 2,3,7,8-tetrachlorodibenzodioxin
TCR-α, T cell receptor-α
TFAs, Trans fatty acids
TG, Triglyceride
TGF, Transforming growth factor
TJs, Tight junctions
TL1A, Tumor necrosis factor–like ligand 1
TLRs, Toll-like receptors
TNBS, 2,4,6-trinitrobenzenesulfonic acid
TNF-□, tumor necrosis factor
TRADD, TNF receptor-1 associated death domain protein
TRAF2, TNF receptor-associated factor 2;
Treg, Regulatory T cells
TZDs, Thiazolidinediones
Ub, Ubiquitin mediated
UC, Ulcerative colitis
UCP-2, Uncoupling protein-2
UPR, Unfolded protein response
VCAM-1, Vascular cellular adhesion molecule-1
VEGF, Vascular endothelial growth factor
X-ALD, X-linked Adrenoleukodystrophy
XBP1, X-box binding protein 1
ZO-1, Zonula occludens-1
α-SMA, Alpha smooth muscle actin

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