REVIEW

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Enhancing Metformin Effects by Adding Gut Microbiota Modulators to Ameliorate the Metabolic Status of Obese, Insulin-Resistant Hosts

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ABSTRACT

Obesity is a systemic disease and represents one of the leading causes of death worldwide by constituting the main risk factor for a series of non-communicable diseases such as type 2 diabetes mellitus (T2DM), cardiovascular diseases and dyslipidemia. Lifestyle interventions have been attempting to prevent T2DM and obesity but are difficult to maintain by most patients. However, the recent focus on the intestinal microbiota and its important role in the host's metabolism provides a new key for improving metabolic health. Modulating the composition of the gut microbiota was proposed as a method to manage these metabolic diseases and most frequently this is undertaken by using probiotics, prebiotics or synbiotics. Furthermore, the action of metformin, the most commonly prescribed drug for treating T2DM, is mediated in part by the gut microbiota, although this interplay may also be responsible for the frequent gastrointestinal adverse effects of metformin. Thus, adding a gut microbiota modulator (GMM), such as probiotics or prebiotics, to metformin therapy could amplify its anti-diabetic effects, while decreasing its adverse reactions. This review summarizes the various therapies that are used to shift the composition of the microbiome and their efficacy in alleviating metabolic parameters, it assesses the interaction between metformin and the gut microbiota, and it evaluates the existing clinical and preclinical studies that analyze the potential synergy of a combined metformin-GMM therapy.

Key words: intestinal microbiota – metformin – probiotics – prebiotics – obesity – insulin resistance – metabolic syndrome.

Abbreviations: Akt: protein kinase B; AMPK: activated protein kinase; AP-1: activator protein-1; ASBT: apical sodium-dependent bile acid transporter; BMI: body mass index; FFAR2/3: free fatty acid receptor 2/3; FGF: fibroblast growth factor; FL: *Flos Lonicera*; FMT: fecal microbiota transplant; FPG: fasting plasma glucose; FXR: farnesoid X receptor; GLP-1: glucagon-like peptide 1; GLUT4: glucose transporter type 4; GMM: gut microbiota modulators; GPR41/43: mammalian G protein-coupled receptor 41/43; GUDCA: glycine-ursodeoxycholic acid; HbA1c: glycated haemoglobin; HC: *Houttuynia cordata*; HOMA-IR: homeostatic model assessment for insulin resistance; hs-CRP: high-sensitivity C-reactive protein; IFN-γ: interferon-gamma; IKK-β: inhibitor of nuclear factor kappa-B kinase subunit beta; IL: interleukin; IRS-1: insulin receptor substrate 1; IR: insulin resistance; JNK1: c-Jun N-terminal kinase 1; LPS: lipopolysaccharide; OFS: oligofructose; OLETF: Otsuka Long-Evans Tokushima Fatty; MOS: konjac mannan-oligosaccharides; mTOR-S6K: mammalian target of rapamycin-ribosomal S6 kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PYY: peptide YY; SB: *Scutellaria baicalensis*; SCFA: short-chain fatty acids; SGLT-1: sodium glucose cotransporter-1; T2DM: type 2 diabetes mellitus; TJ: tight junction; TGR5: Takeda G-protein receptor 5; TLR4: toll-like receptor 4; TNF-α: tumor necrosis factor α.

INTRODUCTION

Obesity and type 2 diabetes (T2DM) are metabolic disorders that have been on the rise, reaching pandemic levels in the past four decades, preponderantly in the industrialized countries and even more so in low and middle-income countries, largely due to unhealthy nutrition, mostly comprised of high-calorie, high-fat, high-refined carbohydrate foods, coupled with sedentarism [1]. These diseases and their complications constitute a huge global economic burden, with an important part of the medical resources focused on their management.

Obesity is a systemic disease, causing complex alterations to every organ, and is associated with insulin resistance

(IR), which is a key element to the development of T2DM, atherometabolic syndrome and dyslipidemia [2]. It is noteworthy that not all obese individuals are insulin-resistant and the association between these two conditions is largely dependent on the distribution pattern of the adipose tissue. Thus, visceral fat positively correlates with IR, while subcutaneous fat is rather protective of IR [3]. Visceral adipose tissue is more metabolically active than subcutaneous fat and can lead to IR through several mechanisms, including secretion of adipokines and inflammatory cytokines, oxidative stress, mitochondrial disfunction and lipotoxicity in the liver and muscle [4]. Recently, another factor incriminated in the pathogenesis of obesity and IR is the gut microbiota.

The human body hosts over 100 trillion microbes and most of them are commensal bacteria that live in symbiosis in the gastrointestinal tract, forming the gut microbiota. Taken together, the genes it encodes constitute the microbiome, which is comprised of more than 150 times the number of genes in the human genome [5]. This allows the microbiota to play an important role in the host's metabolism by enhancing the energy extraction from otherwise indigestible carbohydrates, thus producing short-chain fatty acids (SCFA), fermenting dietary amino acids and regulating bile acid metabolism [6]. In addition, it is essential to the maturation of the immune system and can synthetize bactericidal compounds, such as lactate and acetate [7].

Butyrate, propionate and acetate are the most abundantly produced SCFA, butyrate constituting the main energy source for the epithelial colonocytes. Short-chain fatty acids, especially butyrate, have immunoregulatory and anti-carcinogenic effects by inhibiting histone deacetylases [8], while activating the mammalian target of rapamycin-ribosomal S6 kinase (mTOR-S6K) pathway, thus leading to the differentiation of T-cells to Treg cells which produce interleukin (IL)-17, IL-10 and interferon-gamma (IFN-γ), with anti-inflammatory action [9]. In addition, by binding to G protein-coupled receptor-43/free fatty acid receptor 2 (GPR43/FFAR2) and to G protein-coupled receptor-41/free fatty acid receptor 3 (GPR41/FFAR3), SCFA can suppress the nuclear factor-κB (NF-κB) pathway, further decreasing inflammation [10]. Signaling through GPR43 and GPR41, SCFA can also induce lipolysis in the white adipose tissue [11] and can modulate glucose metabolism by stimulating intestinal gluconeogenesis [12] and by increasing the release of satiety-mediating hormones glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) from the gut [13]. Short-chain fatty acids are also associated with enhanced expression of tight junction (TJ) proteins (zonulin and occludin) that strengthen the intestinal barrier, thus decreasing inflammation [14] (Fig. 1).

Dysbiosis, or reduced microbial diversity coupled with a decrease of commensal bacteria that are replaced by opportunistic microbes, has been frequently incriminated as one of the factors responsible for the metabolic imbalances that lead to T2DM and obesity. Cani et al. [15] were the first to demonstrate a causal relationship between the gut microbiota, metabolic endotoxemia and T2DM [15]. In line with this, high-fat diet seems to promote inflammation by increasing lipopolysaccharide (LPS) translocation. Lipopolysaccharide activates toll-like receptor 4 (TLR4) on the membranes of immune cells, but also of other metabolically active tissues such as muscle, liver, pancreatic and adipose tissue. This activates serin-kinases (IKK-β in the NF-κB pathway and JNK1 in the JNK/AP-1 pathway) that lead to the serine phosphorilation of insulin receptor substrate 1 (IRS-1), impeding its signaling activity and, thus, prompting the development of IR [16-18]. Furthermore, by stimulating the NF-κB pathway, TLR4 activation increases the systemic inflammation by the secretion of IL-1β, IL-6, TNF-α, and high-sensitivity C-reactive protein (hs-CRP) from activated macrophages and can also lead to pancreatic beta-cell disfunction [19-20].

With regards to microbiota composition, most studies found that an increased *Firmicutes:Bacteroidetes* ratio is associated with obesity [21], though there is still controversy on this subject [22]. Less controversial is the beneficial effect the increased abundance of *Akkermansia muciniphila* has on metabolic health, therefore it could be considered a potential therapeutical agent for metabolic disorders [23].

The aims of this review are to briefly summarize the most recent findings concerning the potential therapies that can be employed to modulate the composition of the microbiome, to evaluate the mechanisms behind metformin's action and, most importantly, to analyze the existing scientifical literature regarding the possible synergy between metformin and intestinal microbiota modulators.

MODULATION OF GUT MICROBIOTA IN OBESITY AND INSULIN RESISTANCE

Fecal Microbiota Transplant

One of the most direct methods to change microbiota composition is by replacing the host's microbiome with one from a healthy donor. This technique was developed as an alternative for the treatment of *Clostridioides difficile* infection, where it proved very efficient and generally safe. Following a small clinical study demonstrating that patients with metabolic syndrome can benefit from fecal microbiota transplant (FMT) from lean donors [24] and a case report regarding a non-obese woman receiving FMT from her obese daughter, which presumably led to important and unintentional weight gain [25], several researchers hypothesized that FMT could be used in the treatment of metabolic diseases. Further, transferring fecal microbiota from twins discordant for obesity to germ-free rodents resulted in changes compatible with metabolic syndrome in the group receiving microbiota from the obese twins [26]. Similarly, obese rats receiving intestinal microbiota from obese animals with T2DM showed significant progression towards T2DM, while the rats transplanted with microbiota from healthy animals presented ameliorated metabolic parameters [27]. Interestingly, the metabolic status and microbiota composition of lean animals was not influenced by either transplant, suggesting that a healthy microbiota is more resistant to transient interferences. More recent human studies demonstrated an amelioration of peripheral IR, as well as improved functional characteristics of the microbiome, in patients with metabolic syndrome, after undergoing FMT from healthy donors, although the changes were transitory [28, 29], indicating that, to achieve more sustainable results, either repeated FMT procedures or different measures such as changes in the diet or probiotic and prebiotic supplementation are required.

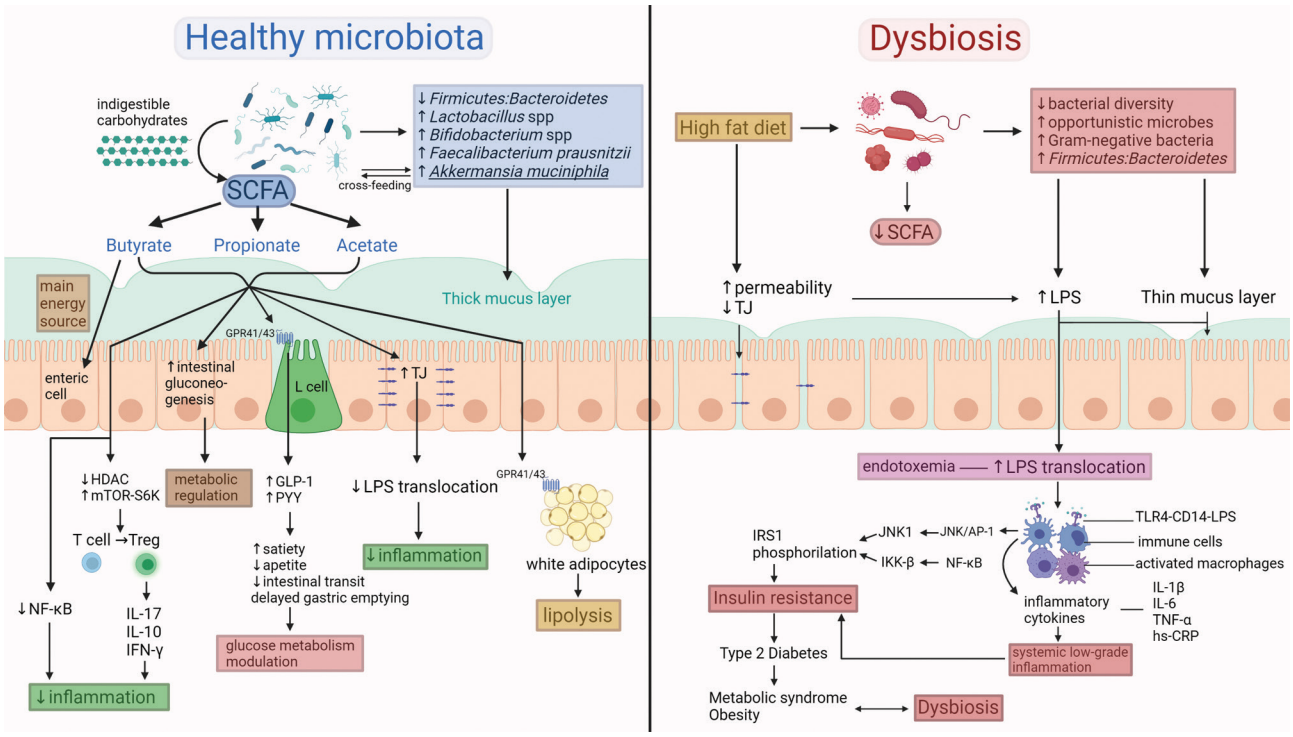

Fig. 1 (created with BioRender.com). The role of the intestinal microbiota in maintaining metabolic health. A healthy and diverse microbial community in the gut can regulate the host's metabolism through several mechanisms. Most importantly, it produces SCFA which constitute a key energy substrate for the colonocytes and the enterocytes and can decrease systemic inflammation by maintaining the integrity of the intestinal barrier and also by modulating pathways related to inflammation (i.e., the suppression of the NF-κB pro-inflammatory pathway and the activation of the mTOR-S6K pathway in the T-cells, with subsequent production of anti-inflammatory cytokines). Additionally, SCFA can regulate glucidic and lipidic metabolism by increasing intestinal gluconeogenesis, stimulating incretin release from the L cells and inducing lipolysis in the white fat tissue. On the other hand, a dysbiotic intestinal environment has a higher abundance of Gram-negative and opportunistic bacteria that produce less SCFA and more LPS. A high-fat diet associated with dysbiosis contributes to increased permeability of the intestinal barrier, allowing the LPS to translocate into the systemic circulation. This triggers an inflammatory response that can lead to insulin-resistance in the liver, muscle and adipose tissues, underlying the development of T2DM, metabolic syndrome and obesity. These, in turn, favor the progression of dysbiosis, thus maintaining a vicious cycle. SCFA: short-chain fatty acids; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; mTOR-S6K: mammalian target of rapamycin-ribosomal S6 kinase; LPS: lipopolysaccharide; T2DM: type 2 diabetes mellitus; CD14: cluster of differentiation 14; GLP-1: glucagon-like peptide 1; GPR41/43: mammalian G protein-coupled receptor 41/43; HDAC: histone deacetylase; hs-CRP: high-sensitivity C-reactive protein; IKK-β: inhibitor of nuclear factor kappa-B kinase subunit beta; IL: interleukin; IFN-γ : interferon-gamma; IRS-1: insulin receptor substrate 1; JNK/AP-1: c-Jun N-terminal kinase/activator protein-1 pathway; JNK1: c-Jun N-terminal kinase 1; PYY: peptide YY; TJ: tight junction; TLR4: toll-like receptor 4; TNF-α: tumor necrosis factor α.

Probiotics, Prebiotics and Synbiotics

A simpler and more accessible alternative to FMT is the administration of probiotics. These are defined by WHO as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [30]. In addition, prebiotics are generally fiber-based dietary components that are indigestible and, instead, can be used by gut microbes as a substrate [31]. Prebiotics and probiotics are simultaneously applied with synbiotics to achieve a synergistic effect. These therapies, also referred to as gut microbiota modulators (GMM), are expected to impact metabolism by effectively modulating the structure of a dysbiotic microbiome towards a more balanced and diverse microbial community. Thus, by promoting bacterial strains associated with metabolic health, such as *Akkermansia muciniphila* or *Faecalibacterium prausnitzii* [32], these therapies could help restore the integrity of the intestinal barrier by increasing the expression of genes that encode TJ proteins [14, 33, 34]. This, in turn, can attenuate traits related to "leaky gut" syndrome and metabolic endotoxemia by decreasing bacterial and LPS translocation, and therefore reduce low-grade systemic inflammation. Other mechanisms behind the effects of probiotics and prebiotics are production of SCFA and anti-microbial molecules, such as lactic acid and hydrogen peroxide [35], enhanced secretion of incretin hormones that mediate satiety [36], immune modulation and cross-feeding the commensal bacteria [37] (Fig. 2).

In recent years, there have been various clinical trials testing the efficacy of GMM in the treatment of metabolic disorders, most frequently T2DM, obesity, metabolic syndrome and dyslipidemia. This reflects in the numerous meta-analyses in the field. Regarding T2DM, out of the 17 meta-analyses identified by us [38-54], with the exception of Kasinska et al. [39], all concur that probiotics or synbiotics can significantly lower fasting plasma glucose (FPG) and most (n=10) also report the ability of probiotics to decrease the homeostatic model assessment for insulin resistance (HOMA-IR), although the reduction of glycated hemoglobin

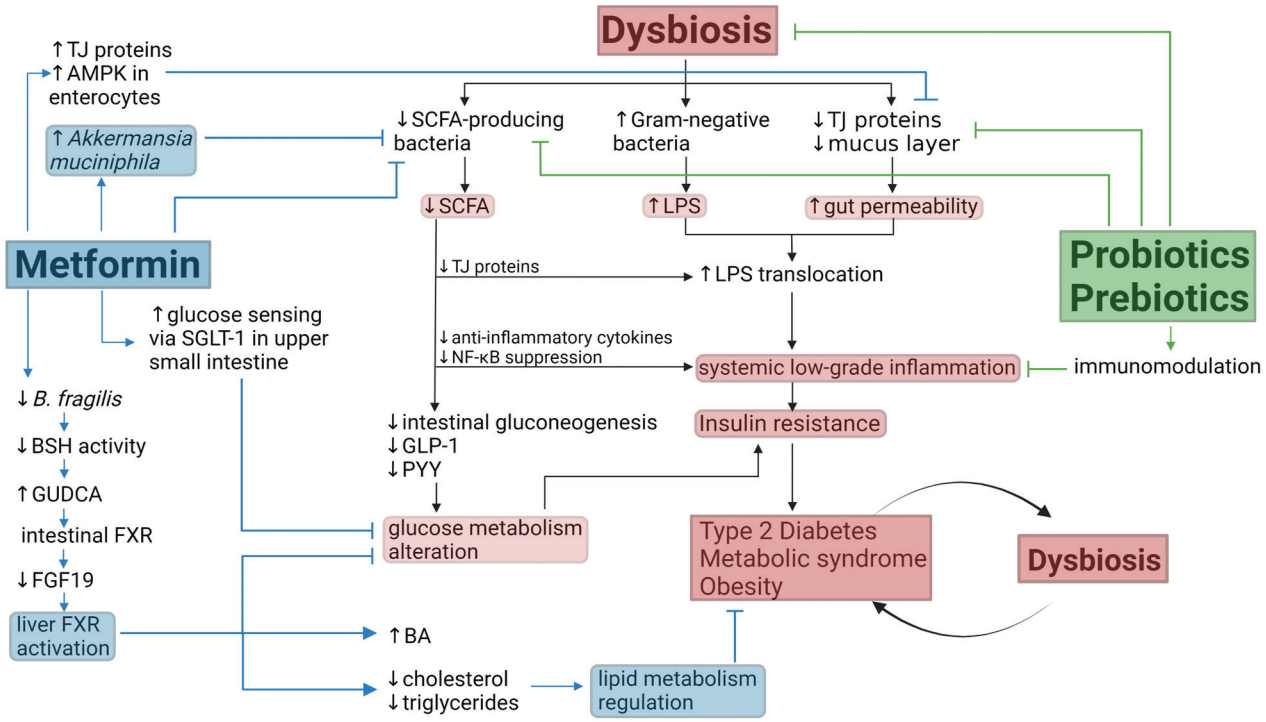

Fig. 2 (created with BioRender.com). The impact of metformin and probiotics/prebiotics on the intestinal microbiota. Metformin and GMM have complementary beneficial effects in the gut, mainly by determining a shift toward SCFA-producing bacteria and an improvement of the intestinal barrier, therefore preventing LPS translocation, inflammation and the subsequent development of insulin resistance. In addition, metformin can ameliorate glucose metabolism by influencing a glucose-sensing pathway mediated by the SGLT-1 in the upper small intestine, while also prompting the release of satiety-mediating hormones from the L cells. Further, metformin can regulate lipidic and glucidic metabolism by increasing the levels of secondary bile acid GUDCA, which inhibits intestinal FXR signaling, while leading to the activation of FXR in the liver. This results in increased production of bile acids and lower cholesterol and triglycerides levels. GMM: gut microbiota modulators; SCFA: short-chain fatty acids; LPS: lipopolysaccharide; SGLT-1: sodium glucose cotransporter-1; GUDCA: glycine-ursodeoxycholic acid; FXR: farnesoid X receptor; AMPK: activated protein kinase; BA: bile acids; *B. fragilis*: *Bacteroides fragilis*; BSH: bile salt hydrolase; FGF19: fibroblast growth factor 19; GLP-1: glucagon-like peptide 1; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PYY: peptide YY; TJ: tight junction.

(HbA1c) (n=8) and fasting plasma insulin (n=8) is less constant. In addition, most underscore the necessity of a longer intervention (usually more than 8 weeks) to achieve positive results and the greater effectiveness of using multiple strain probiotics, while pointing out that the high heterogeneity of the analyzed studies calls for a more standardized protocol for the assessment of probiotics or prebiotics efficacy in treating T2DM. Interestingly, one of the largest meta-analyses on this subject [50], that included 105 articles with 6,826 subjects, indicated that a considerable amount (38%) of the studies they surveyed came from a single country, Iran. These studies had similar designs and outcomes and excluding them from the meta-analyses reduced the anti-diabetic, anti-inflammatory and hypolipidemic effects of probiotics to a non-significant level, thus raising the question whether their results can be applied to all populations. Another important finding of this meta-analysis is the identification of several bacterial species (*Bifidobacterium breve*, *Bifidobacterium longum*, *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus casei* group, *Lactobacillus delbrueckii*) that are more helpful in improving metabolic status and could, therefore, be preponderantly used in probiotic mixtures.

Concerning the benefit of using probiotics to achieve weight loss in obese or overweight patients, several meta-analyses provide even more contradicting evidence, some showing significant body mass index (BMI)-lowering effects of probiotics [50, 55-58], others indicating the opposite [59-61], while Mohammadi et al. [62] found only synbiotics and not probiotics to be effective in decreasing the BMI of obese and overweight children and adolescents. Weight loss after probiotic or synbiotic therapy was also inconstantly reported, some meta-analyses revealing positive effects [50, 57, 58, 61], while others showed non-significant results [56, 59, 60]. Concomitantly, waist and hip circumferences displayed similar variable shifts. In addition, Koutnikova et al. [50] indicated in their meta-analysis that probiotics improved anthropometric indices only in overweight but not obese patients, suggesting that the microbiota of obese hosts could be more resistant to changes induced by probiotics. In general, most of these systematic reviews and meta-analyses agree that more well-designed, homogenous studies that follow standardized protocols are required to clarify which are the bacterial strains and doses that should be used to treat a certain metabolic disorder.

Metformin

Metformin is the most frequently prescribed drug to treat T2DM patients and, even though it was discovered a century ago (1922), its exact mechanisms of action are still

ambiguous. Metformin is considered to act primarily by activating the activated protein kinase (AMPK) in the liver and, thus, decreasing gluconeogenesis, while also increasing the peripheral glucose uptake. Recent evidence indicates that the intestine and, more specifically, the gut microbiota could in fact mediate an important part of its effects.

Firstly, it has been demonstrated that, in mice, intravenous metformin has lower anti-hyperglycemic effects than an identical orally administered dose and it also has no acute glucose lowering effects in humans [63, 64]. Additionally, both animal and human studies revealed that metformin accumulates in the intestinal mucosa, where it can reach up to 300 times the levels present in the plasma [65, 66].

One recently described mechanism behind metformin's modulation of glucose homeostasis is its ability to influence a glucose-sensing pathway involving the sodium glucose cotransporter-1 (SGLT-1) in the upper small intestine by altering the composition of the intestinal microbiota. Transplanting microbiota from metformin-treated rodents fed with high-fat diet to untreated similar rodents, led to the upregulation of SGLT-1 and to an increased abundance of Lactobacillus, proving metformin's capacity to improve glucose sensing while changing microbial composition in the upper small intestine [67].

Another way metformin exerts its anti-hyperglycemic effects via the gastrointestinal tract is by stimulating the secretion of incretin hormones GLP-1 and PYY from the enteroendocrine L cells. Glucagon-like peptide 1 is postprandially released in the circulation and it decreases peak blood glucose by stimulating insulin secretion and suppressing glucagon release from the pancreas [68]. Both GLP-1 and PYY mediate satiety, the first by delaying gastric emptying and both by lowering appetite and food intake [69, 70]. It has been theorized that metformin could directly stimulate the L cells to produce GLP-1 and PYY, though the evidence for this remains scarce and unclear, or it might indirectly raise GLP-1 levels by influencing the bile acid recirculation and the gut microbiome [71].

Animal, human and *in vitro* studies reported that metformin can inhibit bile acid reabsorption from the intestine, most probably by reducing the activity of the apical sodium-dependent bile acid transporter (ASBT) [72-74]. This results in an increased intestinal bile acid pool, which has been associated with the activation of luminal Takeda G-protein receptor 5 (TGR5) in the L cells [75] and the inhibition of nuclear farnesoid X receptor (FXR) [76]. In turn, the activation of TGR5 stimulates the secretion of GLP-1, regulating glucose metabolism [77, 78], and since it has been shown that FXR activation can decrease GLP-1 secretion from the L-cells [79], inhibiting FXR via metformin administration can have the contrary effect on GLP-1 levels. However, evidence suggests that FXR plays an important role in suppressing bacterial overgrowth in the ileum by regulating genes involved in the secretion of antimicrobial compounds and the integrity of the intestinal barrier [80]. Moreover, malabsorption of bile acids leads to chronic diarrhea, therefore, even though the increased intestinal bile acid pool that appears in metformin treatment can have favorable metabolic effects, it might also be responsible for its digestive adverse reactions.

On the other hand, Sun et al. [81] found that, in T2DM patients, metformin treatment can decrease *Bacteroides fragilis* abundance and can thus reduce its bile salt hydrolase activity. As a result, the deconjugation of secondary bile acid glycine-ursodeoxycholic acid (GUDCA) is diminished. They also found that GUDCA acts as an FXR antagonist, therefore increased levels of GUDCA suppress the intestinal FXR signaling, which in turn correlates with lower fibroblast growth factor 19 (FGF19) in humans and with lower fibroblast growth factor 15 (FGF15) in mice. This leads to FXR activation in the liver, which stimulates its production of bile acids, regulates lipid metabolism and improves insulin sensitivity.

More and more studies point out that metformin can induce changes in the gut microbiota at structural and functional levels [82, 83]. To investigate the impact of metformin on the microbiome, in a double-blind trial, Wu et al. [84] randomized recently diagnosed with T2DM, previously untreated patients, to receive either metformin or placebo for 4 months. Whole-genome shotgun sequencing showed significant changes in the relative abundance of over 80 bacterial strains after 2 and 4 months of metformin therapy, while in the placebo group only one strain suffered alteration. Most notably, they reported an increased abundance of *Escherichia* spp. and a reduction of the *Intestinibacter* genus in the metformin group. Interestingly, patients from the placebo group that were later switched to metformin experienced similar changes, with an additional increase of the relative abundance of *Bifidobacterium*. Moreover, after transferring fecal samples from metformin-treated subjects to germ-free mice, improved glucose tolerance of the treated rodents was achieved.

In line with both animal [85] and human [86] studies reporting that metformin promotes higher abundance of mucin-degrading bacteria, Akkermansia muciniphila, Wu et al. [84] confirmed this effect in their study and further demonstrated that, *in vitro*, metformin can directly enhance the growth of *Akkermansia muciniphila* by regulating genes that encode metal-binding proteins. Functionally, metformin therapy was associated with increased production of butyrate and propionate, though only in men, and with higher levels of serum bile acids compared to placebo, which significantly and negatively correlated with HbA1c. These results are consistent with other studies reporting that metformin can enhance SCFA levels by promoting the growth of SCFA-producing bacteria, both in humans [86] and rodents [87]. Thus, metformin also indirectly ameliorates IR by modulating gut microbial composition and raising SCFA levels. These, in turn, improve the intestinal barrier, modulate glucose metabolism through stimulation of incretin secretion from enteroendocrine L cells and have immunoregulatory and anti-inflammatory effects. Furthermore, it has been demonstrated that metformin itself can strengthen the intestinal barrier and decrease LPS translocation by enhancing the expression of TJ proteins in the gut and also by activating AMPK in the enterocytes [88, 89].

However, in a meta-analysis of metagenomic data from Danish, Swedish and Chinese populations, Forslund et al. [90] noticed that, although metformin treatment was associated with higher butyrate and propionate levels, it also significantly correlated in a positive manner with *Escherichia* abundance and it correlated negatively with *Intestinibacter* abundance. These

changes indicate that there might be mechanisms responsible for metformin's beneficial effects, but also for the frequent gastrointestinal adverse reactions of metformin that could benefit from additional microbiota-modulating therapies, such as probiotic, prebiotic or synbiotic supplementation.

METFORMIN & GMM SINERGY

Next, in order to shed light on the hypothesis that metformin and GMM can work in synergism, we will review the animal and human studies that compared metformin therapy and/or probiotics, prebiotics or synbiotics with a conjoined treatment on metabolic parameters of subjects with metabolic diseases, particularly T2DM or obesity.

Animal Studies

In one of the earliest preclinical studies that compared antidiabetic drugs with a functional fiber, in Zucker rats, Reimar et al. [91] demonstrated the superiority of combining a fermentable polysaccharide (PolyGlycopleX) with metformin plus sitagliptin as opposed to administering them separately in improving glucose tolerance, delaying diabetes progression and maintaining the lowest body weight and fat mass and the highest β-cell mass and composite insulin sensitivity index. In addition, serum LPS levels were significantly lower in the group receiving the fiber alone than in all other groups, except the one using the combination of fiber, metformin and sitagliptin. On the other hand, the expression of genes encoding TJ proteins was significantly altered by the drugs, but not the fiber. Therefore, a viscous, functional fiber, with the capacity of slowing glucose absorption, can potentiate the effects of conventional antihyperglycemic drugs, possibly by improving gut barrier integrity and, consequently, by lowering metabolic endotoxemia. However, the impact of these therapies on the composition of the gut microbiota was not assessed in this study.

Another study investigated the effect of adding a probiotic *Bifidobacterium animalis* ssp. *lactis* 420 (B420) to low-dose metformin on diabetic outcomes, in mice with diet-induced diabetes [92]. They found that the low dose of metformin did not affect the glucose tolerance or the FPG, but it significantly lowered insulin levels and HOMA-IR compared to the mice not receiving metformin, while the probiotic had a rather complementary effect, with no impact on plasma insulin and HOMA-IR, instead it significantly lowered FPG. This suggests a possible synergistic effect of combining the probiotic with metformin for diabetes treatment.

In addition, two strains of *Lactobacillus paracasei* subp. *paracasei* G15 and *Lactobacillus. casei* Q14 were used alone or in combination with metformin, in diabetic rats, showing effective restoration of glucose tolerance after 6 weeks of treatment in all experimental groups, though without reaching the normal levels of the non-diabetic control group [93]. Comparable results were achieved in reducing FPG, with the most notable effects observed in the groups receiving metformin and the combination of metformin and *Lactobacillus* Q14, showing the ability of these therapies to ameliorate diabetes outcomes, but not completely reverse T2DM. The researchers noted the alteration of the gut microbiota after inducing diabetes with a high-fat diet, followed by a separate clustering of the microbiota in the intervention groups from the untreated groups. Moreover, after the 6-week intervention, the intestinal permeability was reduced in all treatment groups, as proven by the normalized histological aspect of the colonic mucosa, the significantly lowered LPS and D-lactic acid levels and the subsequent reduction of pro-inflammatory cytokines (IL-1β, IL-8), especially in the combination therapy groups. These aspects further demonstrate the beneficial effects of using probiotics in association with metformin in treating T2DM.

In contrast with the results found in previous studies, no synergistic effects of metformin and probiotic were found in a trial that compared the impact on metabolic parameters of a *Lactobacillus* strain and metformin, alone or in combination, in type 2 diabetic rats [94]. Interestingly, the probiotic exhibited equal anti-diabetic results as metformin and, much like metformin, several mechanisms of the probiotic's action suggested by the authors were the amelioration of leptin levels, insulin sensitivity and glucose uptake by increasing protein kinase B (Akt) phosphorylation and glucose transporter type 4 (GLUT4) expression in skeletal muscles, by lowering systemic inflammation and also by raising adiponectin levels. These results indicate the possibility that not all probiotic strains interact identically with metformin in modifying microbiota composition or metabolic status.

Another study that compared the combination of metformin with oligofructose (OFS), a prebiotic, and their individual administration in diet-induced obese rats, found positive results regarding the amelioration of metabolic parameters [95]. Specifically, weight gain, food intake, body fat percentage, HbA1c, HOMA-IR, glucose intolerance and insulin levels were all significantly decreased in the metformin and the OFS associated with metformin groups compared to placebo group, with the most potent effects being observed in the combination treatment group. The diversity of gut microbiota was decreased under metformin therapy, while adding OFS to metformin gave the opposite result and increased the abundance of bacteria associated with normal metabolic status and intestinal permeability. Moreover, FMT was performed on antibiotic-induced germ-free rats using fecal samples from the treatment groups, resulting in improved glucose tolerance, LPS levels and inflammatory markers in the group receiving transplant from the OFS-metformin treated group. This demonstrates that certain prebiotics could be used to enhance the effects of metformin on metabolic parameters and ameliorate the impact it has on microbiota composition, thus limiting its digestive side effects.

Similar results were observed in a study that analysed the dose-dependent anti-diabetic effects of konjac mannan-oligosaccharides (MOS), which have prebiotic properties [96]. High and medium doses were combined with high and low doses of metformin and compared with single treatments in different doses, in T2DM mice models. The most significant achievements in glycemic control and HOMA-IR reduction were seen in the composite-treatment groups, especially when high doses of MOS were added to high doses of metformin. Also, low doses of metformin had an inferior hypoglycemic effect compared to the same low dose of metformin combined with MOS in a high dose,

thus confirming the cooperation between the actions of this prebiotic and metformin. The results were possibly explained by the protective effect the composite treatment had on pancreatic islets and liver tissue, and also by the increase of microbiota species diversity and the amelioration of carbohydrate metabolism.

Aside from probiotics and prebiotics, other compounds that could have the ability to modulate gut microbiota in order to influence metabolism have been studied, especially in the context of traditional Asian medicine. Most of these are different plant extracts with high phenolic and flavonoid concentrations, that act as anti-inflammatory and antioxidant agents [97]. One such herb extract from *Houttuynia cordata* (HC) has been added to metformin and compared with metformin-therapy alone in Otsuka Long-Evans Tokushima Fatty (OLETF) rats [98]. While both interventions changed the intestinal microbial profile and the production of SCFA, the combination-therapy had significantly stronger insulin-sensitizing and anti-inflammatory effects than metformin alone, lowering both serum and fecal LPS and cytokines like TNF-α and IL-1β, though glucose tolerance was similar in both groups at the end of the experiment. The same authors reported positive effects of this combination in another study on mice with high-fat diet-induced metabolic disorders [99]. The composite treatment, even though it was administered in half the dosage used in the single-therapy groups, was superior to metformin or the HC extract alone in almost all analyzed metabolic, histopathological, inflammation or intestinal microbiota-related parameters, further proving the synergism of a plant extract that has the capacity to modulate gut microbiota and the classic therapy with metformin in restoring metabolic health.

Flos Lonicera (FL), another plant with high flavonoid content, commonly used in Oriental medicine, was added to metformin and compared with metformin alone, in OLETF rats [100]. Similarly, to the HC extract, FL was significantly more effective in improving insulin sensitivity than metformin alone, but it showed almost no difference on glucose tolerance. In addition, lipid metabolism, liver steatosis, oxidative stress and metabolic endotoxemia were ameliorated more strongly in the combination-treatment group than in the metformin group. The alterations of the gut microbiota composition induced by FL further indicate that the addition of a microbiome modulator could be one mechanism through which co-treatment had superior results.

Human Studies

Given the novelty of the topic, studies conducted on human are fewer than animal studies. However, there are several ongoing studies that address the issue [101, 102]. One of the first clinical trials that analyzed the effects of adding a prebiotic blend to metformin showed the combination to be significantly better than placebo plus metformin in alleviating the gastrointestinal symptoms associated with metformin intake, and also in lowering FPG levels [103]. However, it must be noted that this was a very small-sized crossover study, and it did not assess other metabolic parameters, the mechanisms through which the effects were obtained or the changes of the gut microbiome structure.

In another small, single-arm study, T2DM patients on metformin and sulfonylurea therapy were given fiber supplements for 4 weeks [104]. While it reported moderate and inconsistent improvements of IR parameters and increased abundance of some beneficial gut bacteria, these changes were reversed soon after the fiber supplementation was stopped, indicating that only long-term treatment might make an impact on metabolism and microbiota.

Following the proven synergistic effects of *Scutellaria baicalensis* (SB) extract, a plant with prebiotic properties, traditionally used in Oriental medicine, in association with metformin, on metabolic status, in preclinical studies [105, 105], South Korean scientists evaluated in a crossover clinical study the combined effects of either SB or placebo with metformin in T2D patients [107]. Although with SB and metformin a significant amelioration of glucose tolerance, as well as some anti-inflammatory actions were seen, no other improvements of the glucidic or lipidic metabolisms were obtained. Moreover, some of the subjects (most of them in the SB group) presented liver enzymes levels above the normal range after 8 weeks of treatment, though the change was not statistically significant, thus raising the possibility of a mild hepatic toxicity of this therapeutical combination, which warrants further research over longer periods of time. Interestingly, a significant correlation was noticed between some of the results (area under the curve during oral glucose tolerance test, liver enzymes levels) and the baseline compositions of the intestinal microbiota, suggesting that individual gut microbial profiles may lead to different effects of the treatment, therefore raising the prospect of a more personalized approach to these therapies.

Another clinical placebo-controlled study, with a larger sample size, found that only in the subgroup of patients that were on metformin therapy, the multiple-strain probiotic that was used had significant positive metabolic results, by increasing the abundance of butyrate-producing bacteria and, consequently, by strengthening the intestinal barrier [108]. In contrast, when considering the entire cohort, the absence of a notable difference between the probiotic and placebo suggests that the interaction between metformin and the probiotic was responsible for the improved metabolic status.

CONCLUSIONS

Most scientific literature to this date shows promising results regarding the association of metformin and gut microbiota modulators to ameliorate the metabolic status of patients diagnosed with T2DM, obesity or metabolic syndrome. However, the evidence is scarce and more quality studies, following a standardized protocol are warranted to fully elucidate the mechanisms behind this therapeutical interaction and the specific strains and doses that should be used.

Conflicts of interest: None to declare.

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