Chapter 2

# A Network of Physiological Interactions Modulating GI Homeostasis: Probiotics, Inflammasome, mTOR

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**Abstract**

The gastrointestinal surface is in constant interaction with various exogenous molecules. Exogenous components are discriminated in the GI context, as good, in case of nutrients and fibers, and bad, when they negatively affect host integrity. During this tolerogenic process, they also train the host's immune system. The immune system is a morpho-physiologic unit driven by immune cells with the assistance of commensal organisms. Several species of commensal microorganisms have been used for centuries as probiotics due to their beneficial effects on human health. Lowering local levels of pro-inflammatory cytokines has a systemic effect, which is one of the fundamental characteristics associated with probiotics. Still, the primary mechanisms wiring those regulatory circuits as a unit remain unclear. Modulation of the innate immune system, via regulation of inflammasome assembly is emerging as a critical driver of this interaction. Stimulation of toll like receptors (TLR) and inner cell sensors like NLRP3 connect probiotics with essential host systems. In this context, the mTOR-regulated circuits, an intricate network modulating a cascade of protein phosphorylations, could be an important channel connecting host metabolism and probiotics crosstalk.

**Keywords:** *Lactobacillus*, inflammasome, caspase-1, mechanistic target of rapamycin (mTOR), insulin resistance, adipogenesis, type 2 diabetes, cancer

## 1. Overview of probiotics

### 1.1. History and use

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host, as defined by the World Health Organization [1]. This is an extremely

broad definition that encompasses fungal and other eukaryotic species, as well as bacteria. In practice, however, bacterial probiotics receive the most attention. Bacterial probiotics can be found as various supplements and food additives in products such as pills and yogurts [2]. The benefits of probiotic supplements have been recognized for centuries, long before it was understood that the living microorganisms in the supplement provided the benefit. Fermented milk products were used as a treatment for intestinal discomfort in the Roman empire, and ancient Chinese scholars recommended fecal transplant to combat diarrhea [3]. Today, probiotics are often prescribed by gastroenterologists and GI surgeons to help alleviate irritable bowel syndrome, pouchitis, and functional diarrhea, however the potential applications of probiotics in other systems is gaining notice [4]. Yogurt and other fermented milk products as well as probiotic drink mixes are commonly used forms of probiotic supplements today [4].

Strains of the genera *Bifidobacterium* and *Lactobacillus* are the most common bacteria studied and used as probiotics, however *Enterococcus, Streptococcus, Leuconostoc, Bacillus*, and even the yeast *Saccharomyces boulardii* have been used [5, 6]. Knowledge of both the species and strain of bacteria is important in the study and use of probiotics as different strains can produce varying effects on the host. For instance, *Escherichia coli* Nessile 1917 is a beneficial probiotic while *E. coli* 0157:H7 is a deadly pathogen [2, 5]. Sources of probiotics vary. Probiotic bacteria are commonly found in fermented milk products, which lactic acid producing bacteria are essential to the production of, and they have also been isolated from stool samples of healthy individuals [5].

### 1.2. Mechanisms of action

Probiotics can have a wide array of beneficial effects on their host organism (**Figure 1**). One way in which probiotics can benefit the host is to simply prevent or reduce the probability of infection by pathogenic organisms. By forming aggregates with intestinal pathogens, probiotics can reduce the ability of these pathogens to adhere to the intestinal mucosa and initiate infection [7]. *Saccharomyces boulardii*, *Lactobacillus gasseri* 4B2, and *Lactobacillus coryniformis* DSM 20001$^T$ have shown the ability to aggregate with pathogenic strains of *E. coli* (serogroup 0157:H7, and serogroup K88, respectively) [7, 8]. Probiotic bacteria can also increase mucin production in the gut, further reducing ability of pathogens to adhere to and infect host epithelial cells [9]. *E. coli* Nessile 1917 can upregulate the production of MUC2 and MUC3, the primary mucins present in the human colon [10]. Probiotic bacteria often have the ability to produce molecules damaging to pathogens, protecting the host organism by killing or inhibiting the activity of pathogenic bacteria. Several *Lactobacillus* strains produce antimicrobial bacteriocins, some examples include acidocin produced by *Lactobacillus acidophilus*, and sakacin produced by *Lactobacillus sakei* [11, 12]. These molecules may help the host maintain gut homeostasis by regulating the gut bacterial community. Several *Lactobacillus* species can inhibit the growth of *Clostridium difficile* or *C. perfringens* through the production of organic acids, and *Lactobacillus plantarum* LPAL and *Bifidobacterium animalis* ssp. *lactis* BLC1 produce some unknown bactericidal compounds or bacteriocins that inhibit both species [13]. Beneficial gut bacteria can also induce host immune cells to produce defenses against pathogens. Gut bacteria stimulate the production of an antibacterial, peptidoglycan-binding lectin in mice and in humans [14].

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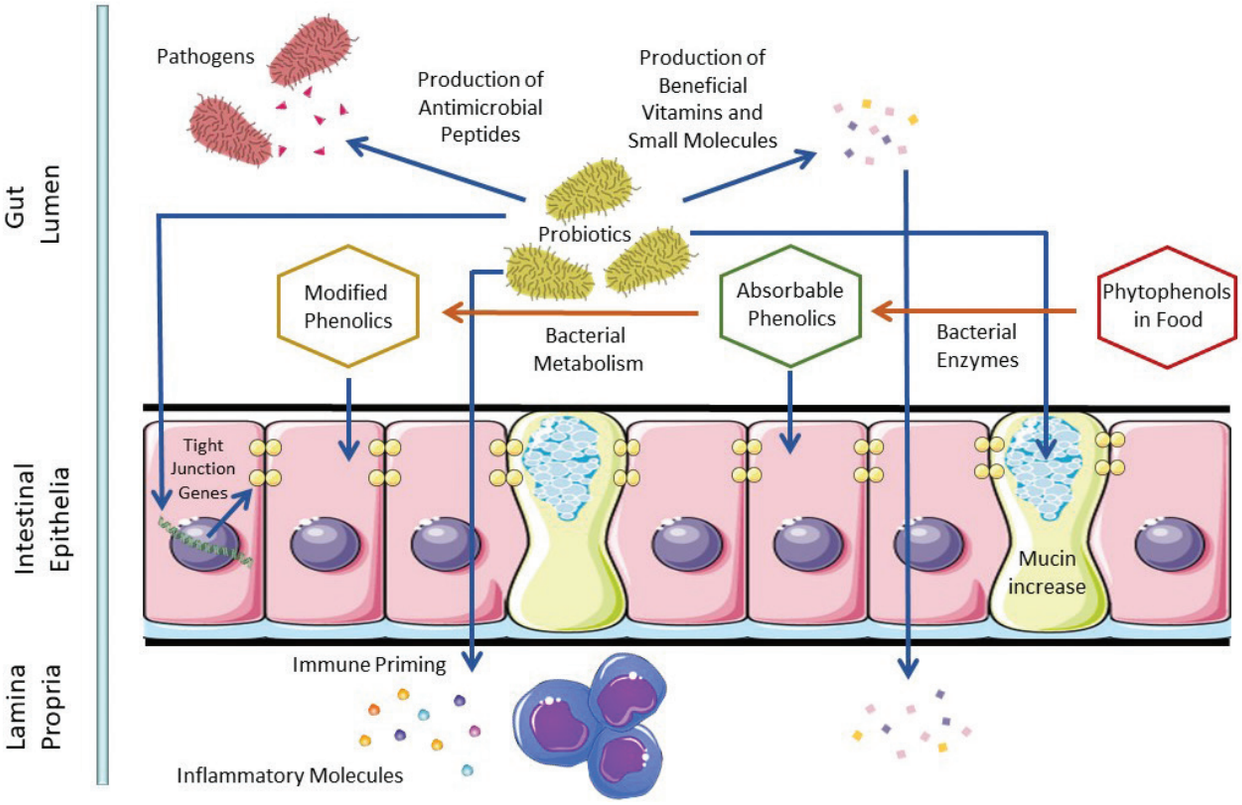


**Figure 1.** Schematic of possible mechanisms of probiotic interactions with molecules in the intestinal lumen as well as host epithelial cells.

Probiotics can also inhibit the growth of pathogens in other ways. *Lactobacillus delbrueckii* can bind iron to its surface, making it unavailable to pathogens, many of which need iron to survive [6]. Probiotics may also benefit the host by reducing the ability of pathogens to diffuse across epithelial cell barriers: strains of *Lactobacillus* show an ability to increase intestinal barrier function. Recent research has documented an increase in the levels of claudin-1 and goblet cells seen in healthy rats as well as in *Lactobacillus johnsonii* fed animals, suggesting that one aspect of the bacteria's role in the gut is to strengthen the barrier function to prevent a leaky gut and maintain a high level of mucin production to protect the gut epithelial cells [15]. *Lactobacillus johnsonii* also appeared to increase the expression of inflammatory chemokines, including CCL20 (MIP3A), CXCL8 (IL-8), and CXCL10 (IP10) [16]. This result may indicate that exposure to beneficial *Lactobacillus* primes the gut immune system so that it is resistant to overwhelming inflammation in the face of later insults [16]. An increase in Paneth cells, immune cells in intestinal crypts, was also demonstrated in *Lactobacillus* fed animals [16]. Overall, probiotic bacteria, many in the genus *Lactobacillus*, can play an important role in defending the gastrointestinal tract from pathogenic organisms.

Probiotics can exert their positive effects on the host by producing vitamins or other materials useful to the host: *Bifidobacterium adolescentis* and *B. pseudocatenulatum* produce B vitamins including B1, B2, B3, B6, B8, B9, and B12 [6]. Probiotics may also increase the availability of nutrients already present in foods. Lactic acid bacteria increase the amount of available folic acid in fermented milk products [9]. The positive effects of Lactobacilli may also result from

the bacterial production of esterases. These enzymes are produced by Lactobacilli and have the ability to release beneficial phenolic compounds, such as ferulic acid and caffeic acid, from food molecules [17]. *Lactobacillus johnsonii* N6.2, a strain associated with diabetes resistance in BioBreeding diabetes prone and diabetes resistant rats, produces two ferulic acid esterases that cleave ethyl ferulate and chlorogenic acid [17]. Other small molecules increased by probiotic bacteria can include free amino acids, and short chain fatty acids such as lactic acid, propionic acid, and butyric acid, which can be used by host cells for energy [9]. Some strains of *Lactobacillus* can produce hydrogen peroxide, which is beneficial to the gastrointestinal tract when present in small amounts [18]. In the case of host lactose intolerance, some strains of lactic acid bacteria, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* can aid in the host's digestion of lactose by supplementing host lactase with their own [9]. *Lactobacillus* species can also increase the nutritional value of various food products. Fermentation with several *Lactobacillus* strains increased the dietary phenol available in cereal grains by a considerable amount [19]. Through both the synthesis and the breakdown of various substances, probiotics can improve host nutrition.

Probiotics have also shown promise in the area of cancer research. *Lactobacillus casei* and *L. rhamnosus* GG can reduce invasion in colon cancer cells, a key property in preventing metastasis [20]. Levels of matrix metalloproteinases, implicated in cell invasion, can be responsive to probiotic treatment: *Lactobacillus acidophilus* and *L. rhamnosus* GG can decrease the expression of matrix metalloproteinase-9 by increasing the expression of the tissue inhibitor of metalloproteinases [20]. Treatment with kefir reduces the viability of colon cancer cell lines by inducing apoptosis and the proliferation of colon cancer cell lines by arresting the cell cycle in the G1 phase [21]. These results suggest that probiotics may be useful in the treatment or prevention of some cancers.

### 1.3. Health benefits

Probiotics are commonly used for gastrointestinal complaints and issues, and there has been extensive research on the benefits of probiotics in this body system. Modern research often supports the old assertions that consumption of probiotics is beneficial to gastrointestinal health. Probiotic supplements have shown efficacy in treating certain intestinal disorders in animal models and in humans. Patients in remission from pouchitis who received probiotic treatment in the form of a bacterial supplement called VSL#3 showed increased *Bifidobacterium* and *Lactobacillus* diversity compared to patients receiving a placebo treatment [22]. *Bifidobacterium* and *Lactobacillus* are commonly regarded as beneficial members of the gut microbiota [23]. VSL#3 was also found to reduce the frequency of pouchitis recurrence [24]. This provides support for the use of probiotics in the treatment of GI diseases. A fermented soy probiotic mixture was shown to provide multiple gastrointestinal health benefits to rats with induced colitis. Rats fed the probiotic mixture of *Bifidobacterium longum* and *Lactobacillus helveticus* 416 had no colon damage, ulcers, or swelling, compared to rats who did not receive the probiotic supplement [25]. The rats receiving the probiotic also showed increased intestinal *Lactobacillus* and *Bifidobacterium* populations [25]. Supplementation with probiotics can help adjust the gut microbiota, and this likely plays a role in the effects of diseases of the

gut. Apple juice fermented with *Lactobacillus* species showed the ability to inhibit *Helicobacter pylori in vitro*, but did not negatively affect other positive GI bacteria [26]. This further shows the ability some probiotics have to ameliorate disease-induced tissue damage and regulate the gut microbiota.

Probiotic supplements are no cure-all for gastrointestinal maladies, however. Assorted studies have reported little to no benefit of probiotics in the treatment of other gastrointestinal diseases. *Lactobacillus* probiotics were not shown to be an effective treatment in helping patients with Crohn's disease stay in remission [27]. In a clinical trial involving women with irritable bowel syndrome, treatment with probiotics was not more effective than the administration of a placebo in reducing IBS symptom severity [28].

On the other hand, there is also a wealth of research showing probiotics to have benefits in areas of the body besides the adult gut. The importance of an individual's microbiome is evident even before birth, therefore the prenatal and neonatal use of probiotics is an important consideration in infant health. The systemic benefits of probiotics can be transferred from mother to infant. In a study on allergies, a probiotic combination taken by an allergic mother, consisting of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12, decreased the probability of sensitization in breastfed infants, possibly by increasing the concentration of the anti-inflammatory cytokine transforming growth factor-beta 2 (TGF-β2) in breast milk [29]. Here we see the ability of a probiotic to induce immune changes in one organism that can be transmitted and positively affect the health of another.

Certain strains of bacteria have also been shown to reduce the negative effects of oral infections. In a study involving mice that were intubated with *Lactobacillus gasseri* SBT2055 and then infected orally with *Porphyromonas gingivalis*, the intubated mice showed less alveolar bone loss and better maintenance of the periodontal ligament than non-intubated mice [30]. In this case, pretreatment with probiotics helped prevent oral damage from infection. Probiotics may also help maintain or improve liver health. A probiotic mixture containing *Bifidobacterium* and *Lactobacillus* species reduced weight gain, maintained intestinal barrier function, and reduced liver inflammation in rats fed an inflammation-inducing high fat diet [31]. Another study using various *Bifidobacterium* strains corroborated these findings. *B. pseudocatenulatum* LI09 and *B. catenulatum* LI10 showed the ability to reduce D-GalN-induced liver damage and serum levels of inflammatory cytokines in rats [32]. Fang et al. found that supplementation with probiotics reduced levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), improved liver necrosis and inflammatory cell infiltration, reduced bacterial translocation to mesenteric lymph nodes, and reduced levels of interleukin 1β, macrophage inflammatory protein 1α, monocyte chemoattractant protein 1, and macrophage colony-stimulating factor in rats [32].

Probiotics have been used for centuries around the globe to improve health and treat disease. Although they are most commonly used to treat gastrointestinal diseases, they can exert positive effects on the health of the entire host organism. Although there has been much research elucidating how probiotics benefit their host and what benefits they actually provide, there is still much to be discovered about the many potential benefits of probiotics.

## 2. The effects of probiotics on the inflammasome

### 2.1. Inflammasome: the interface between detection and response in inflammation

Inflammation is a complex immune response to many different insults, such as pathogens, cell death, and chemicals, which promotes survival during infectious diseases or injuries, as well as maintains tissue homeostasis. When an insult is identified, a cascade of signals is triggered, concluding in the recruitment of neutrophils and macrophages, which have the ability to produce several cytokines and chemokines. Despite the beneficial effects of inflammation, it must be tightly regulated, otherwise it may lead to serious tissue damage due the overproduction of inflammatory cytokines [33]. The secretion of cytokines is regulated at the transcriptional level, and many of them are also regulated at the posttranslational level [34]. Considering that the exposure to pathogens and chemicals is the first step in inflammation, the gastrointestinal environment has a crucial role in this process. Gut epithelial cells are the first cells to be exposed to both microbiota and food components, leading these cells to be key players influenced by food antigens, pathogens, toxins, and also by bodily metabolism and functions. Furthermore, the gut epithelial cells are the first line of defense against pathogens, complementing the action of the associated mucosal immune system, the development and maintenance of which are induced by the microbiota [35]. Some intestinal diseases are largely affected by the gut microbiota, such as inflammatory bowel disease (IBD), and Crohn's disease (CD) [36].

### 2.2. Components of inflammasomes

The mechanisms to identify an insult and trigger an immune response may vary according the kind of the antigenic molecule. In order to identify different antigen molecules, the innate immune cells of mammals can detect these molecules through a fixed number of germline-encoded pattern recognition receptors (PRRs), which have the ability to recognize microbial structures called pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acid and bacterial cell wall [37]. Furthermore, damaged host cells can release some molecules termed danger-associated molecular patterns (DAMPs), such as ATP, reactive oxygen species (ROS) and uric acid, which also have the ability to trigger PRRs [38]. Some PRRs are located in the cell membrane and endosomes and are called toll like receptors (TLRs) and C-type lectin receptors (CLRs), which are able to recognize PAMPs and DAMPs located in the extracellular milieu. The other class of receptors is the NOD-like receptors (NLRs), which are located inside the cell in the cytoplasm [39].

TLRs were first characterized by Christiane Nusslein-Volhard in 1985, when she observed that the protein encoded by *Toll* gene was responsible for preventing the dorsoventral patterning in *Drosophila* embryos [40]. Later, it was observed that TLRs trigger a specific response for different microbes, ending up in the activation of specific regulatory pathways [41]. To date, several TLRs have been classified in mammals and theirs targets identified. Highly conserved, TLRs belong to type 1 transmembrane glycoproteins and are composed of three main structural components: a leucine-rich motif for ligand recognition at N-terminus; a single transmembrane helix; and a cytoplasmic Toll/interleukin-1 (IL-1) receptor domain at C-terminus, as reviewed by Gao and coworkers [42]. TLRs can be expressed on cell membrane, as well as on endosomal membrane. The

TLRs expressed on cell membrane are TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11 and TLR12, whereas TLR3, TLR7, TLR8, TLR9 and TLR13 are expressed on endosomal membrane [43]. Each TLR specifically binds to microbial molecules, triggering a cascade of signals that result in the transcription and production of pro-inflammatory cytokines and chemokines. TLR4 is one of the most studied TLRs due its ability to detect lipopolysaccharide (LPS), leading to the activation of both myeloid differentiation antigen 88 (MyD88)-dependent and MyD88-independent pathways [44]. Downstream, MyD88 is responsible for the activation of the master transcriptional regulators MAPK and NF-κB, which increase transcriptional expression of IL-1β, IL-6, IL-8 and IL-18 [45].

Like TLRs, NLRs can sense different molecules and trigger an inflammatory response. NLRs also have a structure composed of three main domains: caspase recruitment domain (CARD) or pyrin domain (PYR) at N-terminal; the highly conserved NATCH domain, a nucleotide-binding domain (also called as NBD); and leucine-rich repeats (LRR) at the C-terminal [46]. Based on the N-terminal domain, NLRs are subdivided into 8 sub-families (**Figure 2**). The LRRs are responsible for microbial molecule detection, whereas the CARD and PYD domains are responsible for homotypic and heterotypic interactions of NLRs with downstream molecules, such as procaspase, directly or via the adaptor molecule, apoptotic-associated speck like protein (ASC) [47].

### 2.3. Inflammasome assembly

Once epithelial cells recognize PAMPs or DAMPs, many different responses can be triggered in order to eliminate the source of those molecules. One well-known response against pathogens is called the inflammasome. Inflammasomes are a multiprotein complex formed in response to PAMPs and DAMPs, resulting in the activation of caspase-1 (canonical pathways) or caspase-11 (non-canonical pathway) [48]. NLRs located in the cytosol act as sensors of these microbial molecules, leading to the activation of the inflammasome complex. The inflammasome is basically composed by the NLR family members, which may contain the PYR domain or just the CARD, and by the adapter ASC. ASC has both CARD and PYD domains, and the association between ASC and CARD-containing NLRs recruits caspase-1 via homotypic interactions [49]. Despite around 23 NLR genes having been identified to date, only some of them can form oligomeric complexes which end up in the post-translational activation of caspases [50]. The hallmark of the inflammasome is the recruitment of caspase-1 in the canonical pathway, which is further released and subsequently activated via auto cleavage. Active caspase-1 can cleave and activate more than 70 substrates. This sequential process will finally release active caspase-1 to activate the IL-1β cytokine and gasdermin-D to promote adaptive and humoral immunity (**Figure 2**) [51]. In the non-canonical pathway, cleavage and activation of interleukins can also occur via caspase-4/caspase-5 (humans) or caspase-11 (rodents) [52].

Despite the fact that caspase-1 is a protein that plays an important role in many different pathways, one of the most studied ones is pyroptosis, which is a cell death caused by inflammation in response to microbial infections or nonmicrobial stimuli [53]. In pyroptosis, caspase-1 is activated through inflammasome assembly and its active form can then cleave gasdermin-D (GSDMD) at Asp276, which generates the N-terminal cleavage product (GSDMD-NT) triggering pyroptosis and cell death. GSDMD-NT has the ability to form pores on the cell membrane, leading to cell leakage and the release of pro-inflammatory cytokines [54]. Moreover, active

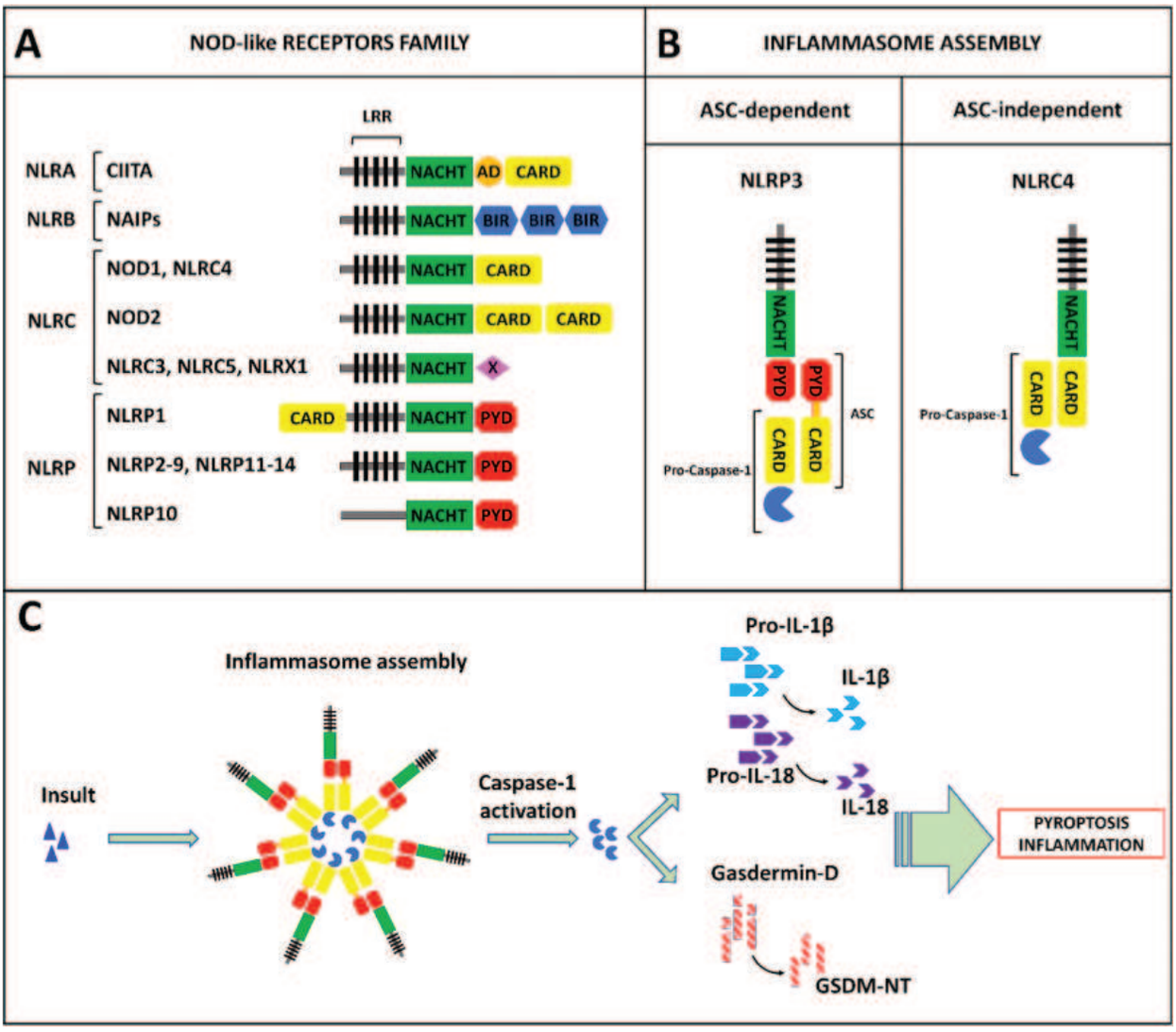


**Figure 2.** NLRs families and triggering of the inflammasome. (A) NRRs currently known families, showing the highly conserved NACHT domain; (B) differences between ASC-dependent and ASC-independent binding to caspase-1; (C) schematic activation of the inflammasome.

caspase-1 can also cleave pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their active form. IL-1β is a pyrogenic cytokine that can promote adaptive and humoral immunity. Neither IL-1β nor IL-18 are secreted by the endoplasmic reticulum-Golgi route. Nevertheless, IL-18 is constitutively expressed in macrophages, whereas IL-1β expression is regulated by NF-κB-mediated transcription [48]. There are other signals that can also trigger the auto-cleavage of pro-caspase-1 independent of NLRP3 activation. Some examples of these secondary signals are ROS and unfolded proteins [55].

### 2.4. Dysbiosis and inflammasomes

The gastrointestinal system harbors a diverse and complex microbial community that has a pivotal role in host health. However, changes in the microbiota population can have major consequences, beneficial or harmful, for host health. The disruption of the gut microbiota, called

dysbiosis, has been observed in several pathological conditions such as obesity, diabetes, and IBD, encompassing ulcerative colitis (UC) and CD [56, 57]. In humans, susceptibility to type 1 diabetes has been associated with changes in the gut microbiota composition, with a significant augmentation of bacteria of the Bacteroidetes phylum, and lower concentrations of *Bifidobacterium, Lactobacillus,* and *Clostridium* strains [58]. The search for probiotic strains that can reestablish host health has strongly increased in the past decades. Most of the microflora of healthy hosts is composed of bacteria from four bacterial phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria [59]. The genus *Lactobacillus* belongs to the Firmicutes phylum, which explains the large amount of studies with *Lactobacillus* species being administered as probiotics.

The inflammasome has been considered as an important regulator of intestinal homeostasis, due the central role of IL-1β and IL-18 in Th1 responses by the induction of IFNγ [60]. Moreover, IL-1β is responsible for induction of neutrophil influx, activation of myeloid cells and lymphocytes, and stimulation of Th17 differentiation [61]. Despite the fact that activation of inflammasomes increases the maturation of the pro-inflammatory cytokines IL-1β and IL-18, there is some evidence that the inflammasomes are important for keeping intestinal homeostasis and reducing morbidity and mortality in dextran sulfate sodium (DSS)-induced colitis in mice. It has been shown that mice deficient in some NLRs, such as NLRP1, NLRP3, NLRP6, NLRP12, AIM2, or deficient in ASC exhibited higher levels of pro-inflammatory mediators, as well as an increase in the epithelial damage within the colon, as reviewed by Chen [60]. Surprisingly, the severity of DSS-induced colitis seems to be reduced when antibiotic therapy is provided to mice, which strongly suggests the role of the gut microbiota in the phenotype of inflammasome-deficient mice [62]. This result was also observed in another experiment where inflammasome-deficient mice were cohoused with wild-type mice or with mothers of the opposite phenotype. After some days living together, an increase in colitis transmissibility through microbial transfer was observed.

The mechanism by which inflammasomes can affect the gut microbiota composition is still unclear. However, the effects of IL-18 on the production of antimicrobial peptides (AMPs) have revealed a possible explanation. IL-18 is able to upregulate the production of AMPs, which is crucial for microbial clearance [63]. *Asc*$^{-/-}$, *caspase-1*$^{-/-}$, *AIM2*$^{-/-}$, *or Nllrp6*$^{-/-}$ mice have shown lower levels of AMPs when compared with WT, but normal levels of specific AMPs are restored after the administration of recombinant IL-18 [60]. Considering that AMPs can be produced to target a specific microbe, the modulation of AMP production can contribute to the abundance of certain bacterial populations. Administration of Ang4, a well characterized AMP, into *Asc*$^{-/-}$ mice changed the overall diversity and community of gut microbiome, but it was still significantly distinct from the WT mice [63]. All these data suggest that despite the activation of the inflammasome increasing the release of pro-inflammatory cytokines, shutting down this pathway also contributes to undesired inflammation. Thus, the modulation of the inflammasome seems to be a key factor in the prevention of exaggerated inflammation.

### 2.5. Probiotics and inflammasome

Many studies have focused on the use of probiotic strains that could avoid or ameliorate inflammation. One promising treatment for IBD is the commercially available probiotic mixture VLS#3,

which is a mixture of eight strains of lactic acid-producing bacteria (*Lactobacillus plantarum, Lactobacillus delbrueckii* subsp. *Bulgaricus, Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis* and *Streptococcus salivarius* subsp. *Thermophilus*). VLS#3 has been shown the ability to ameliorate and prevent colitis in the Il10$^{-/-}$ murine model [64]. The mechanisms by which VLS#3 can reduce intestinal inflammation are still unclear, but several independent results have shown the effects of VLS#3 on the gastrointestinal tract. It was observed that the administration of VLS#3 decreases the biodiversity of the luminal microbiota on TNBS-induced chronic colitis rats [65]. Moreover, TNBS-induced colitis rats treated with VLS#3 have demonstrated pro-inflammatory cytokine and chemokine levels similar to the levels observed in normal rats [66]. These results are in agreement with the effects of VLS#3 observed on the inflammasome of NOD mice: decreasing the mRNA levels of *Il1b* and increasing the mRNA levels of *Ido*, an immunomodulatory enzyme, comparable to control group levels [67]. Surprisingly, VLS#3-treated NOD mice also reduced effective T cells/regulatory T cells (Teff/Treg) ratios at both systemic and pancreatic lymph nodes levels, helping in the maintenance of the immune homeostasis and avoiding excess inflammation.

Due to the proximity of the vaginal mucosa to the gastrointestinal system, the vaginal microbiota is largely affected by the gut microbiota, being dominated by Lactobacilli [68]. Recently, *Lactobacillus rhamnosus* GR-1 has been reported to be able to limit *Escherichia coli*-induced inflammatory response in Bovine Endometrial Epithelial Cells [69]. It was observed that *L. rhamnosus* reduces inflammation by downregulating *Tlr2, Tlr4, Nod1* gene expression, as well as the downregulation of *Myd88* and *Nfkb* mRNA levels. Moreover, this strain showed the ability to reduce mRNA levels of the main components of the inflammasome: NLRP3, ASC, and Caspase-1. Consequently, the mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-18, and TNF$\alpha$ where suppressed by *L. rhamnosus*.

The activation of the inflammasome seems to not be dependent on bacterial viability or require phagocytosis, but the potassium efflux seems to be crucial. A study with bone marrow-derived macrophages (BMDMs) incubated with heat-killed *B. infantis* did not show an increase in the IL-1β levels when compared to cytokine levels when BMDMs were incubated with live bacteria. However, when the cells were incubated with heat-killed bacteria overnight, the IL-1β levels were similar to the levels observed when incubated with live bacteria [70]. In the same work, it was observed that using cytochalasin D, a phagocytosis inhibitor, did not significantly change the IL-1β levels. Interestingly, when WT macrophages were incubated with high concentrations of potassium or with the potassium channel blocker ruthenium red, the levels of IL-1β were significantly lower in response to *B. infantis* or *B. fragilis*, suggesting that the activation of NLRP3 inflammasome is dependent on potassium efflux.

The modulation of the inflammasome by probiotics or gut microbiota does not only affect the gastrointestinal system. In fact, the gut microbiota can modulate the inflammasomes and its effects systemically. The concentrations of pro- and anti-inflammatory cytokines have been correlated with some neurological pathologies, such as depression, which is characterized by high levels of pro-inflammatory cytokines (i.e. IL-1β and IL-6) and low levels of anti-inflammatory cytokines (i.e. IL-4 and IL-10) [71]. Moreover, more IL-1 receptor type-I and its ligands have been found to be highly expressed in brain areas related to stress response, and chronic stress

and the administration of IL-1β have been characterized as triggers of depression-like behavior [72]. Nevertheless, higher levels of caspase-1 and NLRP3 mRNA have been observed in blood cells of depressed patients, which suggests that the inflammasome pathway may play a key role in the development of depression [73]. *Casp1*$^{-/-}$ mice showed decreased depressive and anxiety-like behaviors after a forced swim test compared with WT mice [74]. The effects of chronic restraint stress assay, which increases the caspase-1 and IL-1β levels, also resulted in altered gut microbiota compared to non-stressed mice. The relative abundances of the genera *Allobaculum, Bifidobacterium, Turicibacter, Clostridium,* and the family S24-7 were significantly reduced in restrained animals, whereas the abundance of the family Lachnospiraceae showed an increase. *Bifidobacterium* spp. is a genus associated with the suppression of inflammation by the inhibition of the nuclear factor-κ-B (NF-κB) pathway [75]. All these findings strongly support the notion that the inhibition of caspase-1 can reduce the stress response by modulating the interface between stress and the gut microbiota, and that the gut microbiota can exert some important effects on brain function via the inflammasome signaling.

In the past decade, many studies have demonstrated the effects of the gut microbiota on metabolic diseases. In comparing the gut microbiota of two distinct kinds of rats, the Biobreeding Diabetes Prone (BB-DP) and the Biobreeding Diabetes Resistant (BB-DR) rats, a higher abundance of *Lactobacillus* and *Bifidobacterium* species was identified in BB-DR stool samples [56]. One of the most prevalent species found in this work was *Lactobacillus johnsonii*, which was isolated from the stool of BB-DR rats. *L. johnsonii* has two cinnamoyl esterases that utilizes many phenolic compound as substrates [17]. One well known substrate is rosmarinic acid (RA), a phenolic compound extracted from diverse kinds of plants from the Nepetoideae subfamily of the Lamiaceae family [76]. These cinnamoyl esterases can cleave RA into its two components, caffeic acid (CA) and 3,4-dihydroxyphenylactic acid (DOPAC). Both RA and its components are well known for their antioxidant and anti-inflammatory properties [77, 78]. Based on the activity of the cinnamoyl esterases on RA, a recent study compared the effects of *L. johnsonii* N6.2 when administrated alone or in combination with RA on the inflammasome pathway in the ileum tissue of BB-DP rats fed daily with these treatments. It was observed that, despite higher levels of caspase-1 mRNA and higher levels of pro-caspase-1 in the rats fed with *L. johnsonii* N6.2, this strain decreased the concentration of the active caspase-1, compared to the animals fed with RA alone or in combination with the bacterium [79]. In the same study, it was observed that only RA significantly induced the expression of the *Il1b* gene, 12.5-fold compared to the PBS control. Consequently, RA-fed rats accumulate higher amounts of total IL-1β in the tissue. Lower levels of the pro-inflammatory cytokines TNF*α* and IFNγ were also observed in BB-DP rats fed with *L. johnsonii* N6.2 [80]. A similar result was observed in dogs with chronic enteropathy (CE) that were treated *ex-vivo* and *in-vivo* with *Enterococcus faecium* [81]. It was observed that *ex-vivo* stimulation of duodenal biopsies with *E. faecium* increased the mRNA levels of caspase-1 in CE dogs. However, the protein levels of IL-1β was significantly reduced after treatment. Moreover, *L. johnsonii* N6.2 demonstrated to be able to produce $H_2O_2$, which has an inhibitory effect on the enzyme indoleamine 2,3-dioxygenase (IDO). IDO is the rate-limiting enzyme of tryptophan catabolism, converting tryptophan into *L*-kynurenine. The accumulation of cytotoxic kynurenines due to higher IDO activity can result in localized immunosuppression [82]. All these anti-inflammatory activities

of *L. johnsonii* N6.2 along with its ability to modulate the host immune responses may explain the mitigation of type 1 diabetes in BB-DP rats when fed daily with this bacterium [15, 83].

## 3. Probiotic effects on a master regulatory pathway

### 3.1. mTOR: a master regulator of major cellular functions

Like any living thing, a cell's main goal is to grow, proliferate, and ultimately, survive. This requires the coordination of multiple environmental signals, working synergistically through several pathways in order to culminate into a common outcome. Intricate organization and intracellular crosstalk is necessary for this to be accomplished. Often, these coordinated signals require a regulator to ensure that these functions are carried out efficiently. For most of these processes, the mechanistic target of rapamycin (mTOR) could be considered that important moderator. mTOR is a serine/threonine kinase that presents itself into two distinct complexes: mTORC1 and mTORC2. Ultimately, this pathway integrates external and internal cues to encourage a cell to grow, proliferate, and survive. It senses a diverse set of nutritional and environmental stimuli, including growth factors, amino acids, energy levels, oxygen and stress in order to stimulate anabolic cellular processes like protein and lipid synthesis, and to discourage catabolic processes like autophagy. Deregulation of this pathway has been heavily linked to metabolic disorders and cancer [84].

### 3.2. The mTOR complexes: mTORC1 and mTORC2

As of today, mTORC1 is better characterized out of the two mTOR complexes. This complex is composed of three core proteins and two inhibitory proteins as follows: mTOR, Raptor (regulatory protein associated with mTOR), mLST8 (mammalian lethal with Sec13 protein 8), DEPTOR (DEP domain containing mTOR interacting protein), and PRAS40 (proline-rich AKT substrate of 40kDA) [85]. A popular path to mTORC1 activation is through PI3K/AKT [86]. Here, growth factors and hormones bind to their receptor and activate the intracellular phosphatidylinositide 3-kinase (PI3K) which, through multiple interactions, leads to phosphorylation and partial activation of protein kinase B (AKT). AKT activation phosphorylates and consequently inhibits the tuberous sclerosis complex (TSC). This inactivation stimulates mTOR by inactivating Rheb's (Ras homolog enriched in brain) GTPase domain so that active GTP-bound Rheb binds to mTOR.

Activation of mTORC1 leads to an increase in protein synthesis, lipid biosynthesis, and a decrease in autophagy [85]. Downstream, mTORC1 promotes protein synthesis essentially through two main effectors: p70S6 kinase 1 (S6K1) and eIF4E binding protein (4EBP). S6K1 can also influence lipid biosynthesis by activating the sterol responsive element binding protein (SREBP), which promotes the transcription of genes involved in fatty acid and cholesterol biosynthesis [87]. However, this transcription factor can also be activated by mTORC1, by inhibiting Lipin1, a protein the keeps SREBP localized to the cytoplasm [88]. Peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$), a main regulator of adipogenesis, is also activated by

mTORC1 [89]. Autophagy is inhibited by mTORC1 through the inhibition two main effectors: ULK1 and DAP1 [90]. ULK1 is a kinase that forms a complex with other proteins required for autophagosome formation while DAP1 directly negatively regulates autophagy.

Even though mTORC2 still holds many secrets, we do know a bit about the complex and the functions it regulates. Along with mTOR itself, this second mTOR complex also contains mLST8 and DEPTOR. However, instead of Raptor, mTORC2 contains Rictor (rapamycin-insensitive companion of mTOR), mSIN (mammalian stress-activated map kinase interacting protein 1) and protor 1/2 (protein observed with Rictor 1 and 2) [85]. While mTORC1 is known to be affected by many external stimuli, mTORC2 is resistant to nutrients but is affected by growth factors through a mechanism requiring PI3K. Though this mechanism is poorly understood, it may require the use of ribosomes as ribosomes are needed for mTORC2 activation via a PI3K-dependent process [91].

Not much is known about mTORC2 activation and downstream studies do not hold many answers either. It does seem to primarily control cell survival and proliferation. It is known that when mTORC2 is activated it phosphorylates and fully activates AKT by phosphorylating at serine473 [92]. This mTORC2-dependent phosphorylation unlocks the AKT functions of inhibiting transcription factors FoxO1/3a, which regulates energy metabolism and apoptosis [93]. However, this phosphorylation is not required for AKT inhibition of the TSC complex, therefore mTORC1-dependent functions are not affected. mTORC2 can also directly phosphorylate SGK1, a kinase that controls ion transport and also inhibits FoxO1/3a [94]. Lastly, mTORC2 also regulates cytoskeletal dynamics through the activation of paxillin, PKC-$\alpha$, and Rho GTPases, ultimately affecting cell shape and migration [95].

### 3.3. mTOR deregulation in disease

Since the mTOR pathway is heavily involved in functions affecting survival and growth and responds to growth factors, energy status, amino acids and oxygen, it is not at all surprising that deregulation of this pathway can cause serious systemic problems. Indeed, mTOR is a very complex pathway that seems to play a central role in many fundamental cellular processes. Since new mechanisms of action and regulation are constantly being discovered, it seems that this pathway still has many secrets to be told. Due to the importance of the functions mTOR controls, it is extremely important to keep this pathway in-check. Certainly, this pathway does contain intricate negative feedback loops and inactivating enzymes to prevent the pathway from going into a chronic state of activation. However, like all well-organized systems, a simple flaw could wreak havoc on the system, and there have been plenty of cases reported in disease and research of the consequences that occur in these circumstances.

Since the mTOR pathway integrates glucose homeostasis and lipid synthesis, it is not difficult to believe that disruptions in this pathway can lead to serious metabolic diseases. Indeed, mTOR has been heavily involved in obesity-related comorbidities, such as type 2 diabetes. A high fat diet, a contributing factor to these diseases, has been known to raise insulin, amino acids, and pro-inflammatory cytokines levels, which can affect mTOR activity. Type 2 diabetes occurs when cells become immune to insulin, even when sufficient insulin levels

accumulate to signal cells to take up glucose. mTORC1 has been implicated in regulating the insulin-producing pancreatic β cell function, as β cell-specific TSC component knockout mice revealed that young mice experienced increased β cell mass coordinated with higher insulin levels and increased glucose tolerance [96]. However, as the mice aged, these observations reversed, resulting in a decline of β-cell function over time [97]. This biphasic display could be explained through the feedback inhibition of insulin/PI3K/AKT by constitutive S6K1 expression [98]. At first, constant mTORC1 expression improves β-cell function, however this constant activation eventually accumulates in the S6K1-mediated inhibition of IRS1 upstream of mTORC1. Decrease in β cell function is also observed when mTORC2 signaling is knocked out. In this case, activation of AKT does not occur, which encourages FoxO1 activation. This causes a defect in glucose metabolism, leading to glucose intolerance due to a reduction in β-cell mass and proliferation, affecting insulin production and secretion [99]. It is clear that mTOR is a major regulator in β-cell viability and insulin signaling. Deregulation of this pathway has a great potential to cause insulin resistance leading to diabetes onset.

mTOR signaling also plays a significant role in obesity and non-alcoholic fatty liver disease, both of which can be characterized by an increase in adipogenesis. Fat, the most important energy storage site, accumulates in an mTORC1-activated state, while loss of mTORC1 results in leanness and resistance to high fat diet-induced obesity through enhanced mitochondrial respiration [100, 101]. This is because downstream effectors of mTORC1, 4E-BP and S6K1, regulate adipogenic transcription factors and their translation [102, 103]. Loss of mTORC2, on the other hand, results in impaired glucose transport in response to insulin stimulation and increased lipolysis translating to an escalation in circulating free fatty acids and glycerol [104]. Proliferation of adipose tissue is recognized to be the highest risk factor in developing obesity-related diseases. Over-activation of mTOR has been heavily connected in the tissues of obese and high fat diet-fed animals and its regulation is critical in maintaining a healthy state.

The liver is a multifaceted organ. Not only does it filter and detoxify the blood, it also produces and stores compounds utilized by the whole body. Of importance to this discussion, the liver is responsible for producing triglycerides, cholesterol, and ketone bodies that peripheral organs use as an energy source in low nutrient states. Like adipose tissue and pancreas, mTORC1/S6K1 activity in the liver is high in obese or nutrient dense states, leading to feedback inhibition of IRS and insulin resistance. This inhibition leads to the hyperglycemia and hyperinsulinemia characteristic of type 2 diabetes and insulin resistance. Interestingly, in the liver as well as other tissues, insulin loses its sensitivity yet still retains its ability to stimulate fatty acid synthesis. This could be explained by the fact that FoxO1 in primarily responsible for glucose metabolism in an mTORC2-dependent process, while mTORC1 promotes lipogenesis and this is primarily controlled via SREBP expression [105, 106]. Therefore, this may promote the double-edged sword of glucose intolerance and the stimulation of lipogenic processes, leading to obesity and insulin resistance in an mTOR-dependent manner.

Lastly, imperfect mTOR signaling plays an important role in many cancers. This pathway is made up of many proto-oncogenes and tumor suppressors that, if affected, could turn a cell into a constitutively growing and proliferating state characteristic of cancers. PTEN (phosphatase and tensin homolog) antagonizes the actions of PI3K, which phosphorylates phosphatidylinositol

4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PTEN recycles PIP3 back to PIP2, blocking downstream PI3K/AKT/mTOR signaling. Mutations in this gene blocks the ability of PTEN to recycle PIP3 and deactivate downstream effects, and it has been found to be frequently mutated in human cancers [107]. Another tumor suppressor, TSC1/2, suppresses chronic mTOR activation but, when mutated, can lead to abnormal and unregulated growth [108]. Hyperactivation of mTOR can also happen at the genomic level, as mutations in *MTOR* have also been found in various cancers [109]. Other oncogenic genes, such as *Akt*, *Pi3k*, and *Rheb*, have been described to encourage proliferation and tumor progression. Many of the pathways genes and proteins have been credited with encouraging a proliferative state when manipulated, and since the mTOR pathway is so vast and largely unknown, pinpointing the problem becomes an impossible feat. Even more challenging is finding treatments that are effective and do not have downstream adverse effects.

In response to stimulatory signals, such as insulin and nutrients, the combination of increased adiposity and insulin resistance resulting from chronic mTOR activation is the main driving factors contributing to metabolic disease. To further complicate the scene, genetic mutations or aberrantly functioning proteins can force a cell into a constitutively growing and dividing unit, reminiscent of cancer. Cancer and metabolic disorders are some of the most common diseases in modern times. To contain or prevent the occurrences of these diseases are the topics of many current research and clinical trials. The mTOR pathway coordinates cell growth and environmental conditions through an intercalated network that must adapt to unstable conditions. The complexity of this pathway, the diverse signals it recognizes, and the importance of the functions it regulates makes this a promising, albeit cumbersome, target for therapeutic intervention.

### 3.4. Therapeutic probiotic strategies to modulating mTOR

Although no studies have directly explored the interaction of probiotics with the mTOR pathway, it is likely to surface soon. With the microbiome a popular topic in research in relation to disease onset and now the emergence of mTOR as a main regulator of essential cellular functions whose deregulation is indicated in disease, it would be not all too surprising if a connection could be made between the two. To be able to implement a non-invasive strategy to treat diseases such as cancer or type 2 diabetes would be a huge leap forward in medical technology. Indeed, a main goal in microbiome research is to be able to understand the effects of these microorganisms in the gastrointestinal context, and to dissect their interactions with the host and their environment, including other microbial species and luminal contents. Though many groups have reported on some of the effects of specific microbial species, there is still much left to be discovered. Here, we will consider some of the connections these effects may have with the mTOR pathway (**Figure 3**), and discuss the potential consequences this may have on the host.

In many cases, disease onset is preceded by a systemic inflammatory response. This is also the case in cancer and metabolic disorders. As mentioned, inflammatory cytokines, such as TNF$\alpha$, are known to be potent inducers of mTOR activity. TNF$\alpha$ is known to inhibit TSC1 through its activation of IKK$\beta$, a link that has also been exposed in tumor angiogenesis [110]. A significant elevation of pro-inflammatory cytokines has also been described to be associated with metabolic disorders, such as obesity and type 2 diabetes. As is the case with cancer,

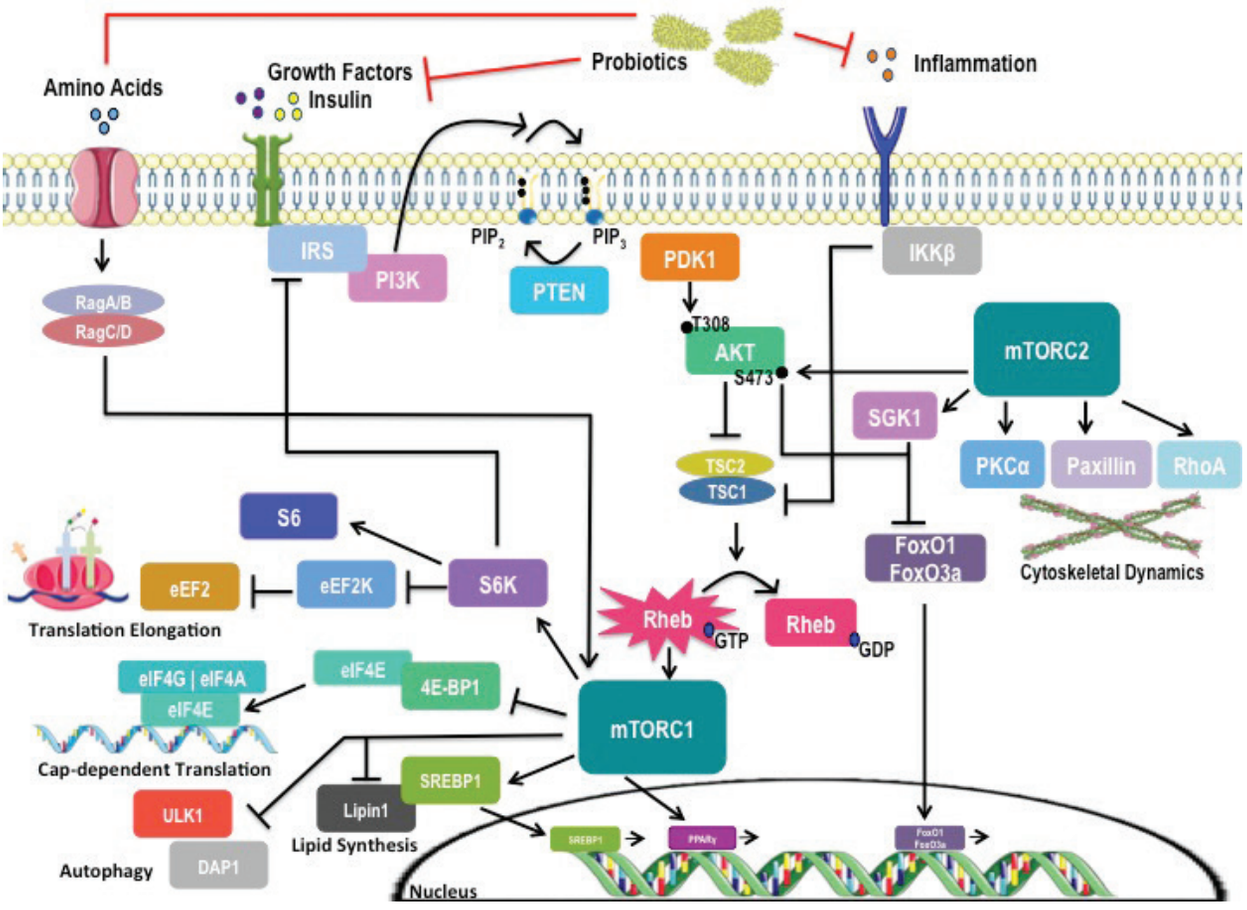


**Figure 3.** A simple representation of the mTOR pathway and some potential probiotic targets. External signals such as inflammation, growth factors, insulin, and amino acids can stimulate mTOR activity through a cascade of upstream effectors. These can activate processes that are required for a cell to grow, proliferate and survive. Using probiotics to target some of these stimulatory signals or enzymes within the pathway can help modulate its effects regarding disease onset.

IKKβ seems to also link inflammation to obesity-induced insulin resistance, and its inhibition could potentially be used to treat insulin resistance [111]. Coincidentally, numerous studies on probiotic strains have focused on alleviating inflammation and have even reported this to be correlated with reduced disease onset [15, 112]. Reducing the circulation of inflammatory cytokines will be less effective in activating IKKβ and therefore stimulating mTOR activity. Therefore, the successful alleviation of inflammatory cytokines with probiotics has the potential to reduce the activation of mTOR and its downstream effects, potentially reducing the incidence of modern diseases associated with chronic mTOR activation.

Insulin resistance occurs when the hormone insulin is insufficient in triggering cells to take in glucose to be converted into energy. Although insulin is produced at reasonable levels, glucose cannot enter the cells and therefore builds up in the blood, leading to hyperglycemia. Both insulin resistance and hyperglycemia are characteristic of type 2 diabetes, and it has been explained that chronic mTOR activation can contribute to this through the negative feedback loop connecting S6K1 to IRS. Obesity and a poor diet have also been described to be risk factors for type 2 diabetes, and associated with mTOR activity. Since the occurrence of type 2

diabetes is dramatically increasing and it continues to be one of the most prevalent diseases threatening human health, there have been many studies commenting on probiotic intervention to reduce symptoms of type 2 diabetes. Several *Bifidobacterium* and *Lactobacillus* strains are described to reducing weight gain, improving insulin-glucose homeostasis and overall improving metabolic syndrome in obese or high fat diet-fed mice [113, 114]. Even a study on a probiotic yeast was found to reduce metabolic syndrome symptoms and hepatic steatosis in obese and diabetic animals [115]. Clinical studies are now investigating this relationship and have reported improved insulin resistance in high fat, over-fed circumstances [116, 117]. However, these studies have rarely looked directly at the mechanism in which these probiotics contribute to human health, and even less often have any investigated into the effects on mTOR. Needless to say, it is possible that these mechanisms could be mTOR-mediated, however more work into this area is needed.

One of the many benefits that our microbial symbionts provide for us is the ability to produce or release substances that our bodies are not capable of doing itself. These substances include vitamins, antimicrobials, butyrate, and other short chain fatty acids (SCFA). In fact, even the famous inhibitor in which the pathway is named after, rapamycin, is produced by the bacterium *Streptomyces hygroscopicus*, providing more evidence that microbes can make specific ligands that interfere with the activity of mTOR enzymes. Studies have elucidated the beneficial effects of butyrate have on colon diseases, such as ulcerative colitis, Crohn's disease, and cancer [118]. To date, two main SCFA signaling mechanisms have been described: the inactivation of histone deacetylases (HDAC) and the stimulation of G-protein-coupled receptors. One study has even uncovered the role of HDAC-activated S6K1 in promoting immune tolerance through T-cell differentiation into effector and regular T cells due to SCFAs [119]. This response is important when cells are faced with a potent stimulus. Instead of over reacting to the stimuli, the T cells emit tolerant signals to be able to neutralize the threat instead of creating a systemic inflammatory response. Additionally, some probiotic strains encode for unique enzymes that can cleave off phenols, or natural antioxidants, from dietary fiber [17, 120, 121]. Coincidentally, many of the inhibitors of the mTOR pathway, such as the popular rapamycin and its derivatives, are cyclic and phenolic in nature. This opens up a new avenue of research, exploring natural food components released by probiotics in controlling pathways whose deregulation is associated with diseases. Few studies have explored this area, but one group discusses the ability of cranberry proanthocyanins to encourage autophagy in esophageal adenocarcinoma cells via inhibition of the PI3K/AKT/mTOR pathway [122]. Another phenolic compound isolated from a shrub is described to disrupt mTORC1 complex and activate the AMPK/TSC signaling cascade, preventing breast tumor growth [123]. Since mTOR activity is aggressive in tumor development, preventing its bodily dissemination through natural food components seems like a far less intrusive procedure than current cancer therapies. Lastly, bacteria can alter the bioavailability of amino acids through their natural metabolism. They can utilize host-derived amino acids, provide amino acids to the host, or disrupt host pathways involved in amino acid digestion or synthesis [80, 124]. Amino acids, particularly arginine and leucine, are essential for mTORC1 activation [125]. Commensals in the intestine have been reported in utilizing these amino acids for protein synthesis, thereby limiting their availability for host-sensitive pathways [126, 127]. However, amino acid producing bacteria within the human intestine can contribute to this available pool of amino acids [128]. The homeostatic maintenance of the bioavailable

pool of amino acids by the gut microbiota may be an important modulator of mTOR activity *in vivo*, thereby controlling disease development. Still, with the emergence of new mTOR data, we are finding that the list of potential inducers of mTOR to be very extensive. Although arginine and leucine are deemed the most important inducers of mTOR, other amino acids have been found to be able to trigger this pathway, and bacteria that have the ability to disrupt host biochemical pathways can regulate this expression [80, 129]. The complexity of the microbial-host relationship in the context of communal metabolites provides an intricate insight into the regulation of important regulatory pathways.

The reduction of mTOR through pharmaceutical intervention has also been a popular area for research. One drawback to this method is that these techniques aim to directly inhibit this pathway through contact with its key mediators. Although, this seems like the simplest and most effective way to prevent mTOR-mediated disease onset, it could create drastic effects. Since this pathway focuses on essential cellular functions, total inhibition of this pathway could do more harm than good. As these drugs are sometimes not natural chemicals, they can also induce unrelated but potentially critical side effects in the body. The best method of action may be to focus on indirect approaches to modulate mTOR activity, rather than trying to completely prevent its activation. These indirect methods could come in the form of moderating its stimulatory signals, such as inflammation and insulin. As we discover more of the health benefits probiotics have to offer, it is clear that this is a multifaceted interaction with the host. After all, these are living organisms, consuming, excreting, and doing what is necessary to survive rather than a chemical that has no consideration of its existence. It is possible that this complex relationship could be what we need to keep our body in balance. Therefore, the answers to relieving some of today's most aggressive diseases could come from our own microflora.

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## Conflict of interest

Authors declare no conflicts of interest.

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