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Review Article

The Ameliorative Role of Probiotics in 5-fluorouracil Induced Intestinal Mucositis

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Abstract: Colorectal cancer (CRC) remains one of the top cancers in the world. Although early detection improves the survival rate to around 90%, late detection would mean the need to use chemotherapy or radiotherapy, especially if surgery is not feasible. 5-fluorouracil (5-FU) is one of the common anti-cancer drugs used in treating CRC. It is the drug that has the

greatest efficacy on CRC. Although it improves the survival rate, it has many detrimental side effects. One of these side effects is intestinal mucositis. It is described as having reduced villus height, villus atrophy, crypt fissure, abdominal discomfort, diarrhoea, and weight loss. Clinically, there is no conclusive treatment therapy for mucositis. This is possibly due to the complex mechanism of the pathobiology of intestinal mucositis that includes the production of pro-inflammatory cytokines and increased epithelial cell apoptosis. 5-FU itself is known to cause gut dysbiosis. Current studies revealed probiotics play a role in attenuating this inflammatory process of intestinal mucositis by reversing gut dysbiosis, reducing the expression of pro-inflammatory cytokines, and reducing intestinal damage. This review outlines the latest evidence supporting probiotic use in ameliorating 5-FU induced intestinal mucositis, thereby promoting good health and well-being in colorectal cancer patients receiving 5-FU chemotherapy.

Keywords: Colorectal cancer; 5-fluorouracil; Intestinal mucositis; Gut dysbiosis; Probiotic; SDG 3 Good health and well-being

1. Introduction

Colorectal cancer (CRC) is the second leading cause of death due to cancer, with 935,000 deaths worldwide[1–3]. The survival rate of CRC depends on the stages of cancer and when it was diagnosed[4]. Generally, 63% of CRC patients will live at least five years post-diagnosis. Early stages can have survival rates up to 90%, while for later stages, especially those diagnosed with metastatic CRC, the survival rates are only around or less than 20%[5,6]. When treating CRC, the goal is to achieve as much total removal of tumours as possible while preserving patients' quality of life and survival. Typically, like most cancers, conventional surgery or chemotherapy is the most common treatment option depending on the size, location, and extent of metastasis. Typically, most early stages CRC patients would undergo tumour resection, while those who cannot undergo surgery would undergo chemotherapy to hinder or kill cancer cells. 5-Fluorouracil (5-FU) is the most common CRC chemotherapy treatment. Currently, 5-FU is either administered as a single drug or in combination with other drugs[7]. Combination regimens available now are the FOLFOX regimen (5-FU and oxaliplatin), FOLFIRI regimen (5-FU, leucovorin, and irinotecan), FOLFOXIRI (5-Fu, oxaliplatin, and irinotecan) and others[7].

Chemotherapy prolongs survival, reduces cancer-related symptoms, and preserves general well-being in advanced CRC patients[8]. Survival rates increase by almost 20% in high-risk CRC patients that undergo chemotherapy[9,10]. Being the backbone of CRC chemotherapy, 5-FU improves overall survival and disease-free interval in stage 3 CRC[10]. 5-FU does this by inhibiting the normal function of DNA and RNA. 5-FU interferes with thymidylate synthesis and inhibits DNA synthesis during DNA replication and repair[11]. Eventually, 5-FU arrests the cell cycle and induces cell death[12]. Although 5-FU is a key drug to treat CRC, it comes with a cost. Since chemotherapy targets tissues with a high cell division rate, other normally dividing cells with the same rate of division are also affected[13].

Repeated treatment with 5-FU, as frequently happens with chemotherapy, 5-FU causes changes to body weight, bowel movement and gut architecture. Weight loss starts around day five post-5-FU, along with diarrhoea two days before. There is around a 10% risk of developing grade three or four diarrhoea (with severe diarrhoea until requiring hospital admission or severe diarrhoea with life-threatening results) in patients that undergo the FOLFOX regimen, and it is doubled in patients receiving FOLFOXIRI[14].

Zooming into the gut's architecture, 5-FU causes villi shortening and reduced number of crypts and crypts cells. Inflammation occurs, with neutrophil infiltration (measured by myeloperoxidase activity), an increase in pro-inflammatory cytokines (PIC) and apoptosis. All these events can be categorised as intestinal mucositis (IM). A common finding is the increased crypt apoptosis and villus atrophy leading to poor nutrition, malnutrition and bacteraemia[15,16]. Having IM is correlated with the application of total parenteral nutrition, analgesics and antibiotics, which are used to decrease the side effects of the inflammation and inflammation. However, in the long run, these are coupled with side effects that impact patients' quality of life, leading to a longer hospital stay, hospital fees and increases in the risk of nosocomial infections[17]. Apart from that, IM often results in dose reduction of chemotherapeutic agents or deferral of treatment, which further increases the mortality rate[18]. All these affect patients' ability to tolerate chemotherapy, affecting their chances of survival[17].

Recognising these challenges, researchers are actively exploring natural resources to offer effective and less toxic alternatives to traditional chemotherapy[19–25], such as 5-FU in this case. The exploration of natural products, particularly those derived from microbes[26–35], animals[36] and plants[37–40], holds promise in improving CRC treatment. While these natural products offer the potential for reduced toxicity and enhanced therapeutic effects, 5-Fu remains the mainstay of chemotherapy used when CRC is at the advanced stage or has a higher risk of recurrence[41]. Among the many therapeutic alternatives, probiotics emerge as the most promising adjuvant in complementing and enhancing the effects of chemotherapy drugs. Probiotics offer several mechanisms through the modulation of the gut microbiome, which plays a crucial role in overall health and immune function[42]. By promoting a healthy gut microbiome, probiotics may improve the body's response to chemotherapy and enhance treatment efficacy[43]. Additionally, probiotics can support gastrointestinal health, alleviate chemotherapy-induced side effects such as diarrhoea, and enhance nutrient absorption[44–46]. By leveraging the symbiotic relationship between the gut microbiome and the body's response to cancer treatment, these studies aim to enhance the effectiveness of chemotherapy while reducing its associated toxicity, ultimately improving patient outcomes and quality of life[44,47].

Gut microbiota influences the responses of a host to chemotherapeutic agents[48,49]. For instance, certain groups of gut microorganisms can enhance drug efficacy, while some promote chemoresistance and mediate chemotherapy-induced side effects[13]. One of the explanations is that chemotherapy causes ROS-mediated DNA and non-DNA damage, which then leads to bacterial translocation through the gut epithelium, hence causing an

inflammatory reaction that can lead to systemic infections and other complications[13]. Unsurprisingly, reports have shown the involvement of gut microbiota in the pathophysiology of IM. Specifically, the crucial role of GM in 5-Fu induced IM has recently been highlighted with the recent study on probiotics, showing that probiotics lessen side effects such as diarrhoea and weight loss, possibly because of restoration of altered gut microbiome composition caused by 5-FU[15]. Therefore, this review discusses the pathophysiology of IM induced by 5-FU and how GM can mediate the inflammatory process. Although most of the studies are performed in animal models, overlapping features are shown in the studies and current understanding of the human body. Key findings in these animal studies would serve as a good fundamental for future human studies. In short, GM can present as a target therapeutic approach in ameliorating chemotherapy-related side effects, especially in patients undergoing the 5-FU regimen.

2. The Role of Gut Microbiome and Their Involvement in The Pathophysiology of 5-FU Induced Intestinal Mucositis

The microbiome is a huge and intricate microbial community comprising bacteria, viruses, archaea, fungi and parasites that inhabit the human body[50]. Among them, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* are the most prevalent phyla of bacteria. There are more than 100 trillion microorganisms in the gastrointestinal tract, which serves as the site of primary communication between the microbiota, the host cells and the host immune system[51,52]. Thus, the gut microbiome (GM) is associated with many functions in the human immune, metabolic, and structural functions. However, the occurrence of gut dysbiosis, specifically the disruption of the balance of beneficial bacteria in the gut microbiome, has been consistently linked to a range of human diseases[53–62]. Recent evidence suggests that certain pathogenic gut microbiota play a role in carcinogenesis[63,64]. For example, sulfidogenic bacteria produce hydrogen sulphide which is capable of damaging DNA. It also inhibits DNA repair mechanisms, leading to extensive DNA damage[65]. These sulfidogenic bacteria include *Fusobacterium*, *Desulfovibrio* and *Bilophila wadsworthia*. Besides that, Gram-positive organisms such as *Streptococcus bovis* or currently named *S. gallolyticus* are positively correlated with the development of adenomas and carcinomas. Various studies show an estimated likelihood of a 6–71% chance of developing CRC in patients that had *S. gallolyticus* bacteremia. Infection with *S. gallolyticus* can occur via infectious endocarditis or the presence of *S. gallolyticus* in the blood[66,67].

IM is a complex process that involves many different cells, tissues and signalling molecules. The pathophysiology of IM is yet fully unravelled. Sonis[68] proposed a 5- step theory consisting of the i) initiation phase, ii) upregulation and message generating phase, iii) amplification and signalling phase, iv) ulcerative phase, and v) the healing phase. It is worth mentioning that the activators for every phase would be a good target for intervention. 5-FU induces dysbiosis in the gut, where it disturbs the delicate balance of homeostasis. Gut dysbiosis is worsened when given antibiotics. Antibiotics kill beneficial bacteria, such as those that secrete metabolites that protect the mucosa and those that influence the metabolism of 5-FU but spare pathogenic bacteria that contribute to decreasing the anti-tumour efficacy

of 5-FU[69]. With this being said, it could be thought that GM is important to humans and may play a role in preventing or treating IM. We will discuss Sonis[68] theory (summarised in Figure 1) with a focus on how the gut microbiome plays a role in this inflammatory process. GM also influences intestinal permeability (IP) and mucus layer, which are the gut's defence barrier.

2.1. Initiation Phase

Based on clinical evidence, the onset of mucositis begins within 24 hours to 48 hours upon the initiation of the cancer treatment[18]. The first stage, which occurs seconds after exposure to chemotherapy, is marked by the production of reactive oxygen species (ROS), direct DNA and non-DNA damage, and immune system activation[13]. It turns out that the human gut is constantly in a steady state of low-grade inflammation. This condition is due to the Toll-like receptors (TLR) on epithelial cells and intestinal-associated immune cells. The conserved molecular motifs on bacteria are recognised by the TLRs outside the cell, activating several intracellular signalling cascades, such as the NF-κB signalling pathway[70]. TLR also maintains homeostasis by preserving the mucosal layer and preventing pathogenic bacteria from disrupting this homeostasis[70].

NF-κB is a transcription factor that upregulates PIC, chemokines and adhesion molecules such as TNF-α, IL-1β and IL-6[68,70]. NF-κB is activated by chemotherapy or radiotherapy and leads to the generation of reactive oxygen species (ROS). ROS are capable of causing tissue damage in the gut epithelium, connective tissue and even blood vessels, eventually resulting in DNA damage and cell death[68]. TLRs trigger a signalling cascade. The importance of TLR can be seen in TLR4 knocked-out mice exhibiting altered neutrophil recruitment and epithelial proliferation. It is thought that TLR4 is able to limit bacterial translocation and modulate intestinal response to injury[71]. This shows that intact TLR signalling might be crucial for the anti-inflammatory response. Whether TLR agonism confers direct protection to either the gut itself or the intestinal microbiome is still unknown. It serves as a potential target site for treating IM as TLRs are frequently exposed to bacteria parts, such as bacterial cell wall.

Due to the constant exposure between TLRs and bacteria, there is always a basal activation of pro-inflammatory transcription factor NF-κB. However, commensals do not trigger an inflammatory cascade[72]. It is postulated that the epithelium sequesters the commensals, preventing TLR activation, while pathogenic bacteria, equipped with virulence factors, are able to bypass the epithelial barrier to be detected by TLR ligands on immune cells[72]. It is also shown that commensals can suppress NF-κB, such as in the case of *Bacteroides thetaiotaomicron* that targets active NF-κB subunit RelA complex and decrease its activation. *B. thetaiotaomicron* also removes NF-κB complexes formed when epithelial cells were induced with virulent *Salmonella* strains resulting in decreased PIC production[73]. The full mechanism of how TLRs are able to differentiate pathogenic and commensal bacteria serves as a gap in present evidence, prompting further research.

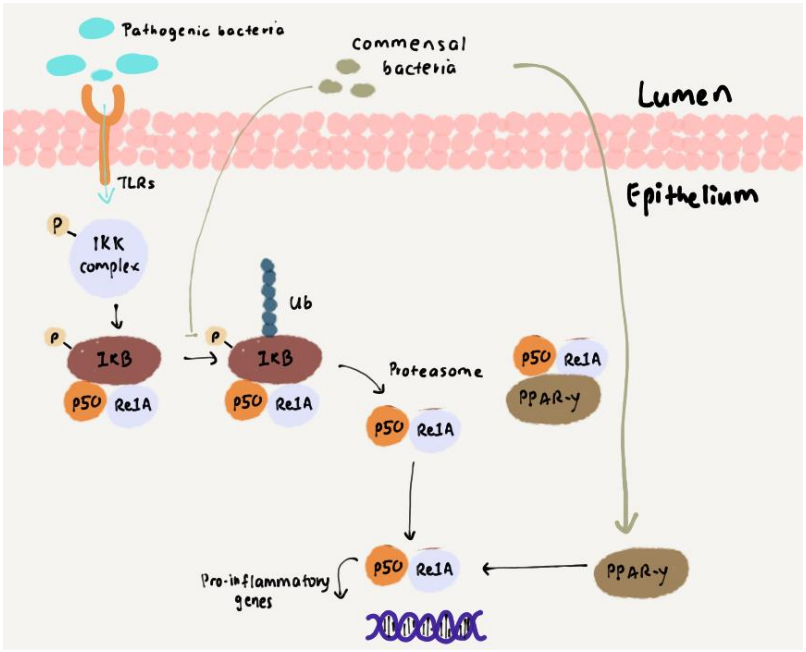

Figure 1. Pathogenic bacteria bind to Toll-like receptors (TLRs). TLRs activate IKK kinase. IKK phosphorylates NF-κB associated proteins, p50 and RelA. The phosphorylated complex is targeted for polyubiquitination, allowing degradation by proteasome, leaving only p50 and RelA. These free proteins translocate into DNA and activate pro-inflammatory gene expression. Commensal bacteria such as nonvirulent *Salmonella* can prevent IKK complex from being ubiquitinated, preventing degradation and incorporation into DNA. Other commensals act at the nuclear level, acting on RelA protein via transcription factor PPAR-γ. These two bind together and dampen NF-κB pro-inflammatory cascade.

Besides *B. thetaiotaomicron*, NF-κB signalling can be suppressed directly by influencing other signalling pathways, as shown by *Bifidobacterium*. IL-10 is an anti-inflammatory, having NF-κB inhibitory properties. IL-10 can also block the transcription of IL-1β, IL-6, IL-8, and tumour necrosis factor (TNF-α)[74]. IL-10 maintains gut immune homeostasis by decreasing PIC concentrations at the tissue damage site[75]. In IL-10 deficient rats, *Bifidobacterium infantis* managed to prevent proinflammatory-associated rearrangement of tight junction (TJ) proteins and instead increase TER. The study also showed that long-term exposure to *B. infantis* reduces inflammation, maintains colon permeability to normal levels and reduces IFN-γ secretion[76]. IFN-γ is an important recruiter for macrophages, leading to immune cell infiltration[77]. Moreover, *Faecalibacterium prausnitzii* secretes an unknown molecule that promotes IL-10 production[78]. Short-chain fatty acids (SCFA) such as butyrates by *F. prausnitzii* can also inhibit NF-κB and IL-2[79,80]. Butyrates are the main products excreted by microbes during the fermentation of dietary fibre (also known as prebiotics). It is found that butyrate suppresses inflammation by reducing TNF-α, IFN-γ, and IL-12 and prevents carcinogenesis by strengthening the mucosal barrier and modulating oxidative stress[81,82]. Probiotic supernatants have immune-modulating properties as well. Supernatant of *Bacillus coagulans* directly increases IL-10 production, which leads to reduced ROS production[11]. Supernatant of *Escherichia coli Nissle* 1917 (EcN) was anti-inflammatory in rats with colitis, reducing TNF-α and increasing IL-10[83]. In short, probiotic is capable of downregulating tissue-damaging ROS by targeting upstream

factors, particularly NF-κB. The exact target to pinpoint in this pathway could be key in reducing IM.

2.2. Upregulation and Message-Generating Phase

After the initiation phase, begins the upregulation and message-generating phase. DNA damage and ROS production activate NF-κB similar to the initiation phase. TNF-α is also activated, leading to epithelial cell apoptosis. NF-κB can transcribe up to 200 genes, most of which are toxic to the gut mucosa. For example, pro-apoptotic proteins are produced by the upregulation of Bcl2 genes and BAX genes. The overall upregulation of genes such as Bcl2 and BAX genes hastens mucosal damage. NF-κB also can increase PIC production in the mucosa. These cytokines damage the connective tissues and endothelium by initiating mesenchymal epithelial signaling and reducing oxygen concentrations in the epithelium resulting in epithelial cell death and injury[68]. This process causes thinning of the mucosa, making it erythematous. This condition can be ameliorated by supplementing with lactic acid bacteria (LAB) such as *Bifidobacterium breve*. LAB protects against inflammation by releasing antimicrobial peptides, IgA. Its presence itself competes with pathogens to adhere to the gut epithelium. LAB have a stronger adherence to epithelial cells by increasing the expression of binding proteins. Besides that, LABs are able to secrete antioxidant enzymes as well[84]. Another study showed *Streptococcus thermophilus* metabolites can cross the mucosa and suppress TNF-α[85].

2.3. Amplification and Signalling Phase

The third phase is the amplification and signalling phase. There is positive feedback by the mediators released in previous phases that magnify other biological factors. Chemotherapy such as 5-Fu can activate NF-κB (a transcription factor), such as found in the gut's epithelial, endothelial, and mesenchymal cells. NF-κB in macrophages can be activated as well. Activation of NF-κB leads to the production of PIC such as TNF-α and interleukin-1β (IL-1β). They participate in positive feedback, further activating NF-κB. NF-κB upregulates transcription of mitogen-activated protein kinase (MAPK), cyclooxygenase 2 (COX2) and tyrosine-kinase signalling molecules.

These biological factors gather in the mucosa and target the tissue. For example, increased TNF-α expression has positive feedback on NF-κB activity. TNF-α can activate caspase 3 (pro-apoptotic protein), which causes cell apoptosis. Upregulation of MAPK also can lead to activation of pro-apoptotic protein caspase 3. Additionally, upregulation of MAPK, COX2 and tyrosine-kinase leads to the activation of matrix metalloproteinases (MMPs) 1 and 3 present in epithelial cells and the lamina propria. MMPs ultimately cause damage to the gut mucosa[86]. Clinically, the gut mucosa may seem erythematous, and patients can manifest very few signs and symptoms while still in the early phases of inflammation[68].

2.4. Ulcerative Phase

The gut barrier consists of the mucus layer, TJ and epithelial cells. Disruption of any of these can impair the barrier. The mucus layer, secreted by goblet cells, provides a physical barrier around the mucosa lining. TJ acts as a seal between epithelial cells comprising a bunch of proteins such as occludin and junctional adhesion molecules. TJ interspersed between epithelial cells is a physical barrier to prevent unwanted substances such as pathogenic bacteria, toxins, or byproducts from penetrating the mucosa. Zonula occludins (ZO-1) tighten the TJ seal at the cell junction[87]. 5-FU decreases both TJ and ZO-1 expression[88]. Upstream inflammatory factors like TNF-α (through NF-κB activation) and IL-1β exacerbate this cycle by down-regulating ZO-1 proteins and altering the localisation of ZO-1 proteins[89,90]. Instead of the normal cohesive binding of ZO-1, it becomes gaplike and zig-zag in appearance. Disruption in both TJ and ZO-1 leads to a penetrable mucosal layer, leading to a more permeable intestine[89].

Besides the connection between epithelial cells, epithelial cells themselves are damaged. Cell apoptosis and villous atrophy occur, forming crypts. Crypts serve as a harbour for pathogenic bacteria to grow on. Crypts also leave nerve endings in the lamina propria exposed, making this phase painful for patients. Besides losing the mucus layer, the epithelial barrier itself is damaged. The gut mucosa is very permeable in this phase, allowing bacteria to translocate across this barrier. The 'leaky' gut mucosa puts patients at risk of developing bacteraemia and sepsis. Direct exposure of the gut to pathogenic bacteria causes the human body to recognise foreign bacteria and start another cascade of inflammatory processes. Mononuclear infiltrates by chemotaxis lead to more inflammation, possibly promoting pro-apoptotic gene expression and PIC, further potentiating further tissue injury (Figure 2)[68].

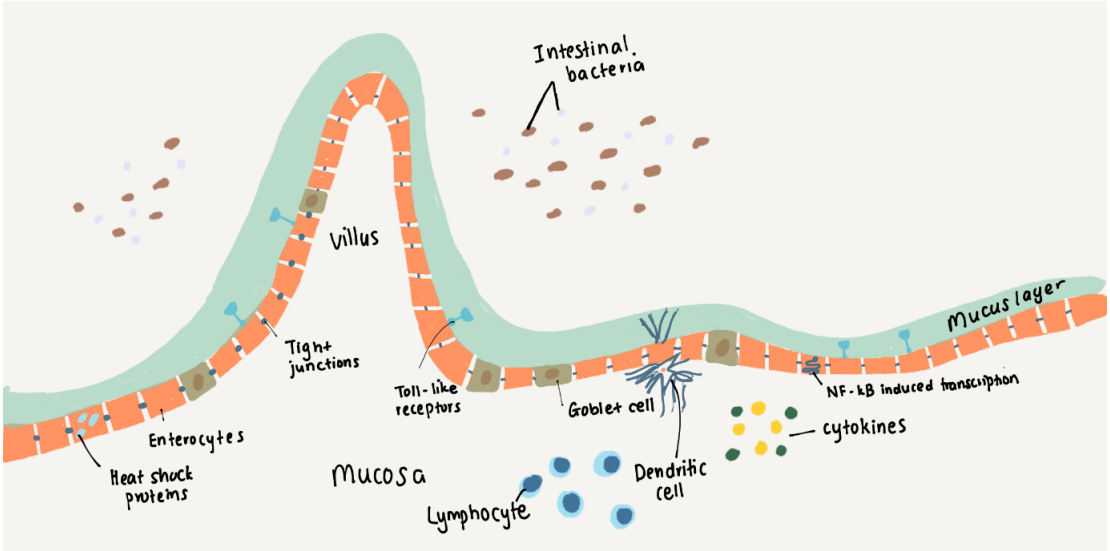

Figure 2. The gut mucosa is protected by one layer of epithelial cells with tight junction interspersed in between cells. This barrier is also reinforced with a mucus layer. Bacteria bind to Toll-like receptors (TLRs). The binding activates NF-κB. NF-κB causes an upregulation of PIC. Bacteria are also phagocytosed by dendritic cells. Dendritic cells present bacteria parts to immunoglobins, subsequently setting off an inflammatory response as well.

Regulating TJ and ZO-1 expression is vital to the progression of intestinal permeability. With the administration of *Bifidobacteria*, there was increased expression TJ forming proteins and decreased intestinal permeability[76]. *Lactobacilli* also has been shown to elevate TJ protein expression and restore intestinal permeability[91–94]. Probiotic strains such as *E. coli* Nissle 1917 and *L. rhamnosus* GG upregulates ZO-1, protecting the gut from being more permeable[95,96]. Upregulation of ZO-1 is important as it protects the epithelial cells from damage such as those caused by pathogenic bacteria like *Escherichia coli* O157:H7[96]. SCFA produced by bacteria is able to reduce IP and increase cell viability[81]. It also helps modulate the intracellular environment, maintaining the intestinal structure, function and integrity and promoting gene expression and proliferation of the epithelial cells[97]. Butyrate, propionate and acetate are usually produced due to the fermentation of undigested carbohydrates or prebiotics by *B. breve* and *Bacteroides ovatus*[98].

2.5. Healing Phase

The last phase is the healing phase. Gut mucosa self-heals itself once the causative factor is removed. In this phase, the extracellular matrix (ECM) releases molecules which promote the healing of the ulcer. First, the molecules migrate from ECM to the ulcer border. Then, migration of the cells occurs to cover the ulcer. Then these cells begin to differentiate into epithelial cells. Normal architecture is then restored. Other molecules that promote hyperplasia are downregulated. Once the ulcer has healed, symptoms of IM begin to resolve[68].

It is known that gut dysbiosis with increased numbers of pathogenic bacteria on an exposed intestinal surface can activate more TLRs. Meanwhile, chemotherapy activates TLRs via NF-κB[70]. It turns out the steady-state inflammation by TLRs and commensal bacteria is proven to allow the repair of the epithelium. Commensal bacteria help to induce the recovery of the gut mucosa. For instance, in a study on chicks, migration of the cells is faster by more than half the time of those in germ-free chicks[99,100]. This allows faster healing of the gut epithelium. In this case, TLR is also significant because it can sense injured or dead cells as TLR recognises and binds to heat shock protein (HSP) produced by dying epithelial cells[70,71]. Cytoprotective effects of HSP are suppressed in TLR knockout mice. For example, a finding indicates that mice deficient in TLR (specifically TLR4) could not repair damaged cells. They experienced more severe damage in the epithelium, with decreased epithelial cell proliferation. TLR4 knockout mice show impaired TLR4 and HSP signalling, delaying response to dead or dying epithelial cells and leading to delayed epithelial healing[71]. As seen before, butyrate is a key player. Butyrate promotes the migration of epithelial cells, thus enhancing mucosal healing[81,101]. Other bacterial products, such as the peptides (p75 and p40) secreted by *L. rhamnosus* GG, prevent epithelial cell apoptosis and stimulate cell growth, hence stimulating the repair of the mucosal layer[102]. *L. lactis* NZ900 producing pancreatitis-associated protein I (PAP) also stimulates epithelial cell growth, including Paneth cells. PAP alters the expression of growth factors and proliferation-related molecules

along with antioxidant enzymes[103]. Figure 3 summarises the 5 phases of mucositis induced by 5-FU.

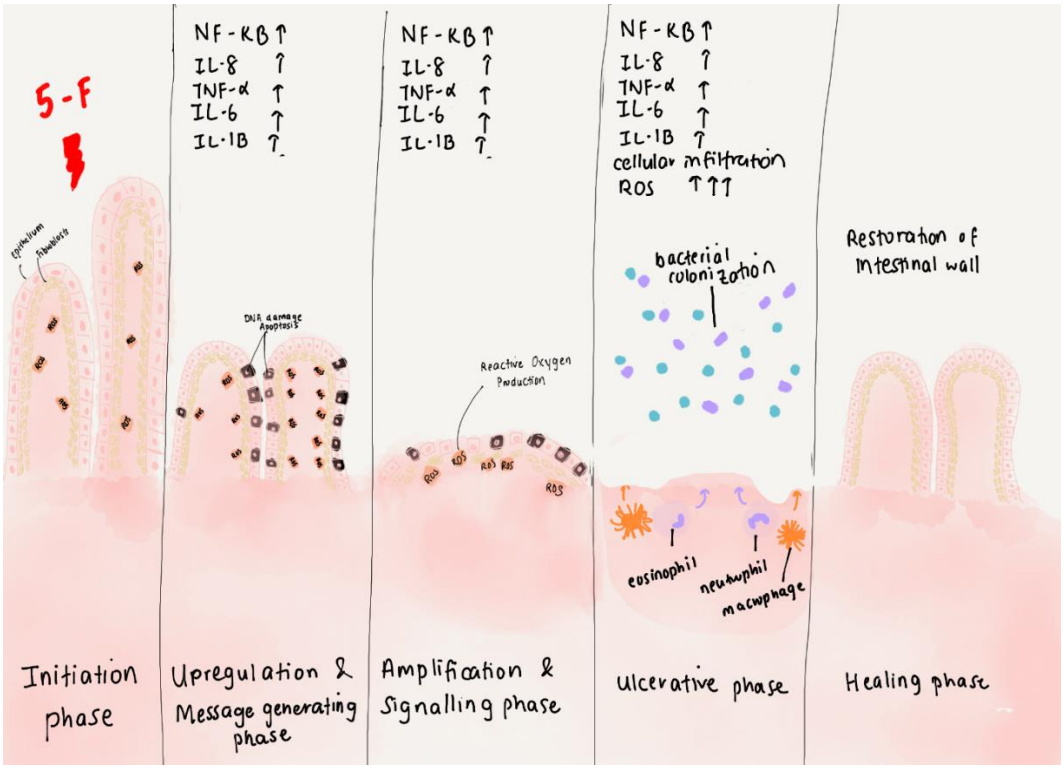

Figure 3. The 5 phases of mucositis. The initiation phase begins with the exposure of gut mucosa to chemotherapy or radiotherapy. Here, we show exposure of the gut to 5-fluorouracil. Reactive oxygen species (ROS) are then formed, promoting DNA damage. ROS activates NF-κB, a transcription factor that subsequently activates PIC, such as interleukin 6 (IL-6), interleukin 8 (IL-8), IL-1β, TNF-α, and cyclooxygenase 2 (COX-2). DNA damage occurs and leads to apoptosis. In the third phase, there is a positive feedback loop in which PIC further reinforce stimulation to produce more PIC, causing further oxidative stress and worsening the injury. Eventually, a vicious cycle is created, and ulcers form due to intestinal injury. After stopping stimuli, the mucosa begins to heal by itself in a few days until the mucosa is restored.

2.6. Effects of 5-FU and Its Impact on Gut Microbiota Composition

5-FU causes a shift in gut microbiota profile by decreasing the abundance of intestine *Firmicutes* while increasing the abundance of *Bacteroidetes* and *Verrucomicrobia*[104]. Specific strains were shown to decrease in abundance, such as *Clostridium spp., Lactobacillus spp. and Streptococcus spp.*, while some increased in abundance, like *Escherichia spp.*[105]. The gut microbiome is in a dynamic state, allowing external factors to influence it, such as antibiotics[106]. Studies have found antibiotics do not offer any clear protection against IM. This was demonstrated by mice that were treated with both 5-FU and a cocktail of antibiotics; pathogenic bacteria such as *Escherichia*, *Shigella*, and *Enterobacter* significantly increased in abundance while other commensals such as *Firmicutes* decreased in abundance. The combination of antibiotics and 5-FU downregulates genes involved in amino acid metabolism, replication and repair translation and nucleotide metabolism, which contributed to overall reduced antitumor efficacy. The effect of GM on 5-FU anti-

tumour efficacy is evident with the administration of antibiotics. Antibiotics use instead worsens patients' body weight in clinical trials. They also showed a higher tumour/body weight ratio in comparison to the 5-FU treatment alone[69]. Therefore, since antibiotics are not working in ameliorating IM, other strategies suitable for use along with 5-FU regimens to ameliorate IM need to be investigated.

3. Gut Microbiota Modulation-Based Strategies to Ameliorate 5-Fu-Induced Intestinal Mucositis

The commensals residing in the human gut are crucial for sustaining a healthy human body. Healthy individuals usually contain bacterial species belonging to four phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*[107,108]. GM helps hosts maintain immunity, protecting hosts from bacterial invasion and nutrient metabolism[47,109]. Shifts in this dynamic structure are present in inflammatory diseases such as inflammatory bowel disease (IBD), psoriasis and multiple sclerosis[107,110,111]. Neurological and psychiatric conditions, such as depression, obsessive compulsive disorder and myasthenia gravis, are also linked to gut microbiome changes[112,113]. Although the full mechanism of all of these interactions has yet to be unravelled, associations between GM and many common illnesses have been evidenced in numerous studies. For instance, gut dysbiosis is also evident in CRC patients. As mentioned earlier, 5-FU and antibiotics are also able to cause shifts in the gut microbial composition. Various strategies such as probiotics, prebiotics, dietary modifications, postbiotics, and fecal microbiota transplantation are utilized for gut microbiota modulation to promote health and manage diseases[114–118]. Among all of these strategies, probiotic use is one of the most common strategies, and it has been shown to prevent or ameliorate dysbiosis of the normal microbiota by maintaining a balanced gut microbiome by reducing potential pathogenic bacteria while increasing the number of beneficial bacteria[119–125]. With that, restoring the disturbed gut microbiota by supplementing probiotics can lessen the side effects of 5-FU, especially IM[126].

3.1. Use of Probiotics in Intestinal Mucositis

Probiotics are "live microorganisms when administered in adequate amounts that confer a health benefit on the host"[97]. They are non-invasive and safe when compared to pathogenic bacteria. Only viable bacteria can be considered probiotics, not including bacteria components that are not viable[18]. Some requirements that must be met to consider a bacterial strain as a probiotic include the ability to adhere to and colonise the gut mucosa to interact with the host to modulate the antagonism against pathogens and for immune system responses[44,127,128]. They colonise the gut temporarily and without releasing toxins or metabolites detrimental to the host. To ensure the viability of the bacteria for colonisation of the intestinal tract, they must be able to resist the environment of low pH and bile salt in the gastrointestinal tract[18]. They help to restore gut dysbiosis by cultivating good commensal bacteria. Probiotics currently are used as adjuvants for many gastrointestinal disorders such as diarrhoea due to infections (Rotavirus infection) or iatrogenic (antibiotic use) or due to other inflammatory diseases such as IBD[12,129]. It is thought that probiotics decrease

pathogenic bacteria that activate the immune system[107]. PIC and ROS have clearly been shown to be the main factors driving the inflammatory process of IM. As such, probiotics can come into play by being an anti-inflammatory agent.

Since it has been shown that commensal organisms play a role in the inflammatory cascade, it would be a good target site to help ameliorate the manifestation of IM. Having said that, not only can probiotics suppress the inflammatory process in IM, but they also promote healing. Probiotics can improve host defence, such as mucus layer, TJ proteins and immune defence, subsequently ameliorating the side effects of 5-FU by reducing apoptosis and improving mucin production. Clinically, weight loss and diarrhoea were also attenuated with the administration of probiotics. Together, probiotics are capable of ameliorating 5-FU induced side effects via different mechanisms[105,130,131].

3.1.1. Gut dysbiosis

The composition of the gut microbiota is altered and modified through a process called dysbiosis due to undergoing chemotherapy, which is often related to the gastrointestinal tract's biochemistry and immunologic disorders[46]. Gut dysbiosis is shown to be common among CRC patients, indicating the possible relationship between the gut microbiome and the carcinogenesis of colorectal cancer[13,132]. A primary increase in Gram-negative bacteria was found following 5-Fu administration in rats, as shown in a preclinical study[13]. At the jejunal level, 5-FU decreases *Clostridium* sp., *Lactobacillus* sp. and *Streptococcus* sp., but increases *Escherichia* sp. In the colon, 5-FU caused decreases in *Enterococcus* sp., *Lactobacillus* sp. and *Streptococcus* sp. Faecal samples showed decreasing trends in *Lactobacillus* sp. and *Bacteroides* sp., and an increasing trend in *E. coli* and *Staphylococcus* sp.[133]. This was also validated in another study of mice administered with 5-Fu showing a shift away from good bacteria such as *Actinobacteria* and towards *Bacteroidetes* and Verrucomicrobia phyla with pro-inflammatory traits[13]. Hence, gut microbiota modulation-based strategies which can shift dysbiosis towards eubiosis or achieve gut microbiota homeostasis should be developed[46]. Probiotics have evidently been able to change this shift in organisms.

Probiotic bacteria that can colonise the intestinal tract have been shown to restore eubiosis by releasing antimicrobial compounds such as bacteriocins and decreasing the pH to limit the development of other harmful bacteria[36]. Gut microbiota is brought back into balance when the amount of pathogenic bacteria reduces due to their inability to survive in the acidic environment while the amount of good bacteria which flourish in the acidic environment increases[18]. This was apparent when researchers used a probiotic mixture in mice and showed a decrease in opportunistic pathogens[69]. In male mice that received both 5-FU and *Lactobacillus reuteri* DSM 17938 (BG), it was found *Bacteroides* and *Bacteroidaceae* were most bountiful in 5-FU treated rats supplemented with the mixture, while those that received only saline solution showed that Muribaculaceae (S24_7) was most abundant[134]. Similarly, *Lactobacillus plantarum* 299v reduced 5-FU stimulated the growth of facultative anaerobes in the intestine[135]. Both probiotic strains, *Lactobacillus casei*

variety *rhamnosus* (Lcr35) and *Bifidobacterium bifidum* G9-1 (BBG9-1), also can significantly reverse the perturbed microbiota composition of *Firmicutes* and *Bacteroidetes* induced by FOLFOX. However, supplementation of Lcr35 itself did not affect faecal GM composition[12,131]. This shows that probiotics are strain-dependent. Hence, further research must be carried out in the future to find out more functional probiotic strains. Another strain, *Rhodotorula mucilaginosa* UFMGCB 18377, was also found to be able to reduce intestinal enterobacteria levels in 5-Fu treated mice with induced mucositis[136]. *In vitro* studies, non-recombinant *L. lactis* NZ9000 showed antagonistic activity against the invasive gut pathogen *Listeria monocytogenes* and its secreted compound called PAP had an inhibitory effect against opportunistic *Enterococcus faecalis*[137,138]. PAP is not the only property possessed by *L. lactis* NZ9000; it is thought that its antagonistic actions against pathogens could also be due to the secretion of lactic acid, bacteriocins or metabolites[137].

3.1.2. Pro-inflammatory cytokines

In the pathobiology of IM, TNF-α is a key PIC that causes IM. Other cytokines involved include interleukin (IL)-4, IL-6, IL-10 and IL-17. IL-17 is an inflammatory mediator. It acts together with IFN-y to synergistically enhance IL-8 secretion by human fetal intestinal epithelial cells[139]. When supplemented with probiotics Lcr35 and *Bifidobacterium bifidum* (LaBi, Infloran®), levels of PIC decreased[12,140,141]. The study shows Lcr35 has the ability to suppress NF-κB activity, also leading to suppression of inflammation and subsequently reduced IM in the intestine[12].

Other *Lactobacillus* strains, such as *Lactobacillus delbrueckii* CIDCA 133 (pExu:*hsp65*), a recombinant strain of *L. delbrueckii* is able to increase serum IL-10 in ileum tissue while reducing inflammatory infiltrate and cytokines such as TNF-α, IL-6, IL-12, and IL-1β[142]. Other strains *S. thermophilus* CRL 808 decreases IL-6 but increases IL-10. This vitamin-overproducing strain can reverse the rise of the anti-/proinflammatory cytokines ratio. It increases concentrations of IL-10 in the serum along with decreased IFN-γ levels[143]. In another experiment on a mouse model, it was found that the levels of TNF-α, IL-1β and IL-6 were higher in the 5-Fu induced mouse group than the control group. However, those concentrations markedly decrease in the use of *S. thermophilus* ST4 along with 5-Fu treatment[97].

B. infantis also successfully reduced high levels of PIC caused by 5-FU in CRC rats. As mentioned, IFN-γ recruits macrophages[77]. IL-2 is important for IFN-γ stimulation while IL-12 stimulates production of IFN-γ by naïve T cells[144] . In this case, it is found that Th1 cells and their cytokines, IL-2, IL-12 and IFN-γ, which are upregulated during chemotherapy, can be reversed by *B. infantis*[145].

3.1.3. Intestinal damage

As mentioned before, changes in the architecture of the intestinal mucosa are side effects of 5-Fu, including a decrease in villus high, an increase in crypt depth and intestinal permeability and a shortening of the intestinal length. Villus blunting and crypts fissure in the small intestine are mainly caused by an increase in apoptosis and a decrease in cell proliferation as a result of chemotherapy[97]. Restoration of the intestinal structure is very important as it influences the ability of nutrients to be absorbed by the body. A taller villus has more surface area for the absorption of nutrients, and it also lessens water loss and electrolyte loss[146]. Inefficient nutrient absorption can lead to loss of weight (LOW) in cancer patients[142]. Hence, probiotics have been proven to play a role in restoring intestinal damage.

In an *in vivo* experiment, *S. thermophilus* ST4 was proven to be able to ameliorate the shortening of colon length of mice treated with 5-Fu. This is shown by a shorter average colon length in the mice treated only with 5-Fu when compared to the 5-Fu + *S. thermophilus* ST4 group. It was also found that the administration of *S. thermophilus* ST4 had also mitigated the decrease in the villus heights and crypt depths[97]. Another probiotic strain, *R. mucilaginosa* UFMGCB 18377, was also demonstrated to be able to decrease intestinal shortening of mice induced with mucositis by 5-Fu from around 17% shortening compared to the control group to 7.5% only. It is also found that this probiotic strain is also efficient in ameliorating the structure of the damaged intestinal mucosa, restoring its architecture and villus to crypt ratio, and reducing the increasing permeability of the intestine as a result of 5-Fu treatment[136].

Mucosal damage, such as crypt depths, has been shown to be significantly restored, even to levels similar to those only injected with saline, with Lcr35 and LaBi supplementation. Villus height to crypt depth ratio managed to return to normal[140], although another study by Huang *et al.*[141] has shown similar results without it being statistically significant. Other strains also showed similar results, with *Saccharomyces boulardii* and *Lactobacillus fermentum* BR11[130,147]. *S. boulardii* also induced the recovery of intestinal permeability by normalising the lactulose: mannitol ratio[130].

Effects of supernatant and live *S. thermophilus* TH-4 reduced ileal crypt fissure by at least half of the controls, although it unsuccessfully ameliorated 5-FU induced mucositis[148]. *Lactobacillus plantarum* ATCC 8014 showed a potential beneficial effect on ameliorating 5-Fu induced microscopic changes in the intestinal mucosa[149]. *Lactobacillus delbrueckii (pExu:hsp65)*, *L. delbrueckii* subsp. *Lactis* CIDCA 133 strain, *L. acidophilus* or milk fermented with *L. rhamnosus* FLRH93 all were able to stop the shortening of the gut due to 5-FU. These probiotic strains also reduced 5-FU's adverse effects on IP, crypt depth, villus height, as well as villus/crypt ratio and goblet cells[142,146,150–152]. *L. rhamnosus* FLRH93 has also managed to prevent small intestine shortening, with an almost 5% difference compared to mice with 5-FU alone. This is consistent with the finding from the same study that the mice had less LOW[150].

Two tested probiotic mixtures, such as *Lactobacillus acidophilus* and *B. lactis,* or a mix of four strains (*Lactobacillus acidophilus*, *Lactobacillus paracasei*, *L. rhamnosus*, and *B. lactis)* partially reversed histopathological changes induced by 5-FU[153]. BBG9-1 as mentioned before, prevented the increase in crypts quantity and reduction of small intestine length (although not statistically significant) but could not stop the induction of crypt cell apoptosis, as shown with *B. infantis* supplementation[145,154].

Supernatants from *E. coli* Nissle 1917 and *F. prausnitzii* enhanced barrier integrity by normalising crypt depth and prevented the change in barrier by 5-FU. *S. thermophilus* CRL 808 and *S. thermophilus* CRL 415 are strains that can produce folate, which was shown to induce less inflammation in the jejunum and preserved villus height/crypt depth ratio[143]. Improvement in the histopathological score was also apparent with *P. freudenreichii* WT[155]. Treatment with *Saccharomyces cerevisiae* UFMG A-905 successfully re-established a normal state in the intestine. Intestinal permeability returned to normal. *S. cerevisiae* UFMG A-905 has antioxidant and anti-inflammatory properties. Inflammatory markers were further reduced after giving selenium-enriched yeast[156].

3.1.4. Immune cells infiltration

The infiltration of inflammatory cells is broadly related to mucosal damage[136]. Neutrophil infiltration, associated with intestinal damage and its recruitment, can be measured quantitatively by myeloperoxidase activity (MPO). In a recent study, 5-FU can increase this by almost 500 times with an 83% decrease in sucrase[157]. On the other hand, eosinophil peroxidase (EPO) activity can be used to quantify the accumulation of another inflammatory cell called eosinophils. Eosinophils play an important role in inducing inflammation in the intestine by acting as antibacterials through the production of PIC and EPO activity. 5-Fu induced mucositis often results in the recruitment of neutrophil and eosinophil infiltrates or causing an increase in MPO and EPO levels, hence triggering inflammatory reactions in the tissues[136]. In a mucositis mouse model, MPO level has been shown to decrease with *L. delbrueckii* subsp. *Lactis* CIDCA 133 strain, *S. boulardii*, *Lactococcus lactis* NZ9000, *Lactobacillus acidophilus* A4 (A4), BBG9-1, *R. mucilaginosa* UFMGCB 18377 and probiotic mixtures[130,131,136,137,151,153,158,159]. *R. mucilaginosa* UFMGCB 18377 can also reduce EPO levels in the ileum and jejunum of mice with induced mucositis due to 5-Fu[136]. Lower MPO and EPO levels also indicate that there is lesser immune cell infiltration, which will reduce inflammatory responses. Besides, fewer polymorphonuclear cells in the lamina propria lead to less inflammatory activity by these cells, following fewer lesions occurring[137]. *L. delbrueckii* subsp. *Lactis* CIDCA 133 has also been shown to minimise 5-FU induced leukopenia[151].

Intestinal microbiota plays a part in the adaptive immune system by regulating IgA secretion. IgA is secreted by immune cells scattered across the mucosa[142]. IgA prevents bacteria colonisation and invasion into the mucosa. Toxic materials are blocked by IgA from penetrating the mucosa. IgA modulates the inflammatory response by inhibiting inflammatory response by activating regulatory T-cells[160]. Clearly, serum IgA has a dual

function in modulating microorganisms present in the gut. It protects the gut from pathogens but also regulates good bacteria. It seems that IgA has a different affinity for different microorganisms. There is a high affinity for pathogenic microorganisms but a low affinity for commensal bacteria[161]. IgA and commensals also regulate each other. *Bifidobacteria* is capable of modulating the expression of secretory IgA. Serum IgA was increased after 3 days of *Bifidobacteria* supplementation in rats that suffered burn injuries in the gut, but these levels were returned to normal by day 5. This self-modulating expression reduced incidences of bacteria translocated and reduced counts of *E. coli* and fungi[162]. Clearly, strains that increase IgA is a good probiotic to consider as well. *L. delbrueckii* CIDCA 133 (pExu:*hsp65*), a recombinant strain can also increase serum IgA[142].

3.1.5. Apoptosis

5-FU is capable of inducing cell apoptosis by inducing the expression of IL-1β and TNF-α[15]. This is shown by its antagonist, interleukin-1 receptor (IL-1R) antagonist, in successfully reducing apoptosis after chemotherapy. Up-regulation of Bax and caspase 3 (pro-apoptotic proteins) along with downregulation of Bcl-2 and Bcl-xL (antiapoptotic proteins) occur during 5-FU induced intestinal apoptosis[163]. Caspases and Bcl-2 proteins are important in early apoptosis[164]. Bcl-2 can prevent cells from harsh environments such as radiation, heat and chemotherapy. Bcl-2 prevent these cells from undergoing apoptosis[163]. *In vitro* study shows that IL-1R antagonist inhibits apoptosis without affecting its anti-tumour efficacy. This shows that antagonising IL-1β activation can protect the gut from chemotherapy-induced IM[163].

Lcr35 has been shown to suppress apoptosis caused by FOLFOX administration. The reduction of the increased BAX/BCL-2 ratio induced by FOLFOX and a shift toward anti-apoptosis when Lcr35 is given[12]. Besides that, *S. boulardii*, a probiotic yeast, has been shown to reduce 5-FU induced intestinal cell apoptosis. 5-FU modifies the TLR response to activate PIC. When this cascade is activated, there is phosphorylation of ERK1/2, p38 and JNK. In particular, TNF-α and IL-1β activate these protein kinases in response to oxidative stress. The IL-1β induced MyD88 pathway and phosphorylation of protein kinases are inhibited by *S. boulardii*[165]. In another case, *the* probiotic *Propionibacterium freudenreichii* requires SlpB protein to alleviate mucositis induced by chemotherapy[130].

3.1.6. Mucin production

The human gut mucosa is protected by a mucus layer. This mucus layer comprises water, glycoproteins, trefoil factors, defensins and mucins. Mucin is an important component, it being the first-line defence in the gut. Mucins are produced by goblet cells that reside all over the small and large intestines and produce mucin[166]. Bacteria are able to adhere to mucin oligosaccharides. Commensals can compete with pathogens to bind and colonise the mucin, preventing pathogens from attaching to the mucosal surface. For example, the parasite *Entamoeba histolytica* requires contact with epithelial cells in order to invade it. Having a thick mucus layer prevents it from having contact with the epithelium. Once this mucus layer

is disrupted, *E. histolytica* can easily penetrate through the gut barrier leading to phagocytosis of epithelial cells[167]. Our gut epithelium is endlessly exposed to various noxious chemicals and physical insults such as digestive enzymes, faecal material, resident bacteria, and intestinal pathogens and their products. Mucin blocks the mucosa from bacterial enzymes by acting as a substrate for enzymes such as α-galactosidase, β-*N*-acetylgalactosaminidase, sialidase, β-glucuronidase, blood group degrading enzymes, and proteases[166]. Having a mucosal shield as a barrier between underlying epithelium and these substances is essential in preventing IM.

The mucus layer is also thickened in response to the presence of pathogens. This quick response is key to eliminating pathogens. In other words, pathogens and their toxins have a positive stimulation on mucin production. Thick mucus allows easier excretion of pathogens but also carries a risk of diminished mucin[167,168]. *Vibrio cholerae* enterotoxin stimulated mucin production[169]. However, *Helicobacter pylori* downregulate mucin-producing genes MUC1 and MUC5AC resulting in reduced mucin synthesis[170]. Another important gene is MUC2. MUC2 is a key player in the formation of the mucus layer. It is stored in goblet cells and is key in determining goblet cell shape[171]. In MUC2 knockout mice, defective or depleted mucus defence layer showed bacteria colonisation and inflammation (with multiple crypts), subsequently leading to carcinogenesis[168,171]. For example, *Lactobacillus plantarum* 299v, *L. rhamnosus* strain GG and *L acidophilus* strain DDS-1 are shown to increase gene expression of MUC2 and MUC3 at the mRNA level[172,173]. This managed to prevent adherence of pathogenic *E. coli* onto gut epithelium[172]. Besides, non-pathogenic commensal *E. coli* Nissle 1917 regulates multiple genes. Besides MUC2 and MUC3, it also upregulates MUC5AC and MUC5A[174]. Butyrate-producing organisms also have shown abilities to increase MUC2 gene expression in *in vitro* studies. Human colon biopsies in *ex vivo* studies showed increased mucous secretion in the presence of butyrate-producing organisms. Trefoil factors, a contributor to the viscosity and elasticity of mucus, can reduce the chemotaxis of inflammatory cells and are thought to repair damaged mucosa. Like mucin, it is only produced by GC. Trefoil factors are reduced in rats with colitis, but with butyrate administration, these levels increased[175].

5-FU treatment has been shown to influence the mucus layer and decrease goblet cells while increasing the number of crypts in the jejunum[176]. This is possibly due to the pro-inflammatory state 5-FU induces. It is found that *Rhodotorula mucilaginosa* UFMGCB 18377 decreases the loss of goblet cells in mice induced with mucositis by 5-Fu[136]. Other than that, treatment with *L. delbrueckii* rCIDCA 133:Hsp65 also increases goblet cells and expression of the MUC2 gene. A higher quantity of goblet cells and increased MUC2 gene expression can reinforce each other's positive effect in protecting the mucus layer[142].

3.1.7. Weight loss

Regarding weight loss, probiotic strains such as Lcr35 and LaBi have been shown to reduce body weight loss by 20%. Mice in the Lcr35 probiotic group showed a lesser degree of LOW compared to 5-FU and saline group[177]. Other *Lactobacillus* strains, such as *L.*

plantarum 299v, have been shown to improve food intake, resulting in less LOW, while others, such as *L. delbrueckii* subsp. *Lactis* CIDCA 133 strain and *L. acidophilus* could increase food and milk intake but still reduce LOW[135,142,151]. Riboflavin overproducing *L. plantarum* CRL2130 also showed similar results[152,178].

Besides these, *L. fermentum* BR11 and *L. rhamnosus* GG increase colon weight in conjunction with 5-FU treatment[157]. *Lactobacillus plantarum* 299v and BBG9-1, at a concentration of 1×10^9 CFU/mL, improved both food consumption and body weight post 5-FU administration[131,147]. Interestingly, high doses resulted in LOW, while low doses resulted in body weight gain after one week of 5-FU treatment. On day 8 after the treatment, low doses increased body weight compared to high doses of the probiotic, which decreased food intake[147]. *B. infanti*s also has shown weight-gaining properties[145,154].

In another *in vivo* experiment in mice, it was found that mice with 5-Fu induced IM showed a significant decrease of about 50% in food intake and about a 20% decrease in their body weight in relation to the initial weight on the 18th day. However, in the 5-Fu + *S. thermophilus* ST4 mice group, there was no big difference in body weight loss (only 1.85%) and food intake compared to the control group[97]. Another strain of probiotic, *Rhodotorula mucilaginosa* UFMGCB 18377 ameliorated the decrease in food consumption of mice induced with mucositis by 5-Fu hence reducing the weight loss in comparison to their initial weight from 20.3% to 12.4% in the probiotic-treated group as compared to the mucositis group[136]. A probiotic mixture (*L. acidophilus*, *L. paracasei*, *L. rhamnosus*, and *B. lactis)* reduced body weight loss[153]. However, the addition of *Streptococcus* genus, resulting in a mixture of *B. breve* DM8310 + *L. acidophilus* DM8302, *L. casei* DM8121 + *S. thermophilus* DM8309 showed no differences in faecal output, and food intake[158]. *E. coli* Nissle 1917 supernatant and *F. prausnitzii* supernatant prevented further weight loss from happening post 5-FU induction resulting in normal urine and faecal output[179].

These mixed results show that, clinically, some probiotic strains are better than others at preventing weight loss. None of the probiotics mentioned exacerbated weight loss, suggesting its importance in maintaining patients' weight. Further work should be done to understand the underlying mechanisms exhibited by some strains to preserve the body weight, potentially via improved feeding behaviour and the host's metabolism[180].

3.1.8. Diarrhoea

Severe diarrhoea is presumed to be closely related to the shortened colon, one of the side effects of the administration of 5-Fu[97]. Diarrhoea occurs when the colon's absorption capacity is surpassed by the rising fluid volume out of the small intestine[44]. Rat studies show that probiotics can ameliorate diarrhoea. The research was carried out on a mice model by assessing the severity of diarrhoea using Bowen's score system, in which diarrhoea is categorised into 4 grades based on the consistency of stool (0 — normal stool; 1 — mild diarrhoea, the stool is slightly soft and wet; 2 — moderate diarrhoea, stool are wet and unformed; 3 — severe diarrhoea, stool are watery). It is found that the 5-Fu + *S. thermophilus*

ST4 group shows a lower mean diarrhoea score that changed from 1.0 to 0.1 when compared to the group of mice induced with 5-Fu only that scored 2.5 and 2.5 respectively, on the most severe day 14th to 15th[97]. For example, Lcr35 group and LaBi group, *L. delbrueckii* CIDCA 133 (pExu:*hsp65*), *L. plantarum* CRL2130, BBG9-1 and also folate-producing *S. thermophilus* CRL 808 were shown to successfully reduce the incidence of diarrhoea[140,143,178].

Probiotics reduced the incidence of diarrhoea in human patients. In a systematic review, researchers found probiotics and fibre reduced the incidence of grade 3 or 4 abdominal discomfort (flatulence, borborygmi and abdominal distension) and the need for chemotherapy dose reduction, compared with placebo[14,181]. *L. rhamnosus* GG decreased the incidence of diarrhoea in grades 3 or 4 by around 15%. In patients with severe diarrhoea, metabolic and nutritional imbalances may occur, which may further worsen their condition. It is evident that probiotics show improvement in diarrhoea in animal models and humans[45]. In another clinical trial on patients treated with 5-Fu based chemotherapy and administered with probiotic *Lactobacillus kefiri* LKF01, it was found that the incidence of diarrhoea was reduced. Only 4.7% and 8.7% of patients treated with 5-Fu and FOLFOXIRI respectively developed G3-4 severe diarrhoea [CTCAE grade [G] 3-4]. In contrast, no incidence of high-grade diarrhoea was reported by patients treated with FOLFOX and FOLFIRI, respectively. Since their results also showed that the onset of diarrhoea mostly started at the early stages of chemotherapy, hence it is hypothesised that the intake of probiotics earlier before the treatment begins and not concomitantly as they did in their studies could possibly work as a preventive measure to reduce the rates of early-onset diarrhoea, but further studies are required to validate it[44].

Table 1. Probiotic genus, strains and mixtures that show beneficial effects toward IM. Different probiotics ameliorate IM in various aspects are summarised in this table.

Probiotic	Effects of probiotic on ameliorating intestinal mucositis	Reference
Lactobacillus acidophilus	• Preserved villus and crypt length ratio • Reduced GSH levels • Reduced myeloperoxidase activity • Reduced nitrite levels • Reduced level of PIC, TNF-α, IL-1β • Reduced levels of chemokines CXCL-1 • Increased levels of anti-inflammatory cytokines IL-10	[152]
Lactobacillus acidophilus A4	• Prevented attachment of *Escherichia coli* O157:H7 • Reduced level of PIC, IL-8, TNF-α, IL-1β • Induced the expression of MUC2	[182]
Lactobacillus brevis 47	• Partially restored expression of Ki-67 epithelial proliferation cell marker	[183]
Lactobacillus plantarum CRL2130	• Reduced weight loss • Reduced diarrhoea scores • Maintained mucosal architecture • Reduced mucosal inflammation	[178]

Probiotic	Effects of probiotic on ameliorating intestinal mucositis	Reference
	• Preserved villus and crypt length • Reduced level of PIC, IL-10	
Lactobacillus plantarum supernatant	• Increased sensitivity of colorectal cancer cells to 5-fluorouracil • Inhibited CD44 gene expression • Inhibited CD133 gene expression • Inhibited CD166 gene expression • Inhibited ALDH1 gene expression • Increased caspase 3 activity • Inhibited signalling pathway Wnt/β-catenin	[43]
Lactobacillus casei variety *rhamnosus* (L cr35)	• Reduced diarrhoea scores • Reduced loss of weight • Normalised crypt depth • Preserved villus and crypt length ratio • Reduced villous inflammation • Diminished apoptosis • Reduced level of PIC, TNF-α and IL-6 • Reduced levels of NF-κB-, and BAX-activated cells • Restored composition of fecal gut microbiota • Decreased *Firmicutes* and *Bacteroidetes* ratio • Reduced CD3+/CD8+ count • Increased CD3+CD4+/CD3+CD8+ • Reduced CD44 the number of Ki67 proliferative cells	[177]
Lactobacillus delbrueckii CIDCA 133 (pExu:hsp65)	• Reduced inflammatory infiltrate • Reduced intestinal permeability • Increased serum IgA in the intestinal fluid • Improved histological score • Prevented shortening of the intestine • Preserved villus and crypt length ratio • Reduced myeloperoxidase activity • Reduced level of PIC, TNF-α, IL-6, IL-12, IL-1β • Reduced level of Toll-like receptors • Increased level of anti-inflammatory cytokines IL-10 • Increased gene expression of MUC2 • Increased gene expression of claudin 1 • Increased the number of goblet cells	[142]
Lactococcus lactis NZ9000	• Prevented growth of *Listeria monocytogenes* • Pancreas-associated peptide secretion prevented growth of *E. faecalis* • Improved histological score • Reduced infiltration of neutrophils • Reduced infiltration of eosinophils • Reduced secretion of Immunoglobulin-A in the gut • Reduced gut inflammation	[137]
Lactobacillus sp.	• Induced expression of MUC3	[173]
Lactobacillus rhamnosus FLRH93	• Preserved villus and crypt length ratio • Lessen the decrease of goblet cells	[150]

Probiotic	Effects of probiotic on ameliorating intestinal mucositis	Reference
	• Increased the expression Bcl-2 in the intestinal tract • Decreased expression of NLRP3 • Reduced level of PIC, TNF-α, IL-1β • Increased survival rate of mice	
Lactobacillus rhamnosus GG	• Reduced diarrhea scores • Reduced incidence of abdominal discomfort	[45]
Streptococcus thermophilus CRL 808	• Enhanced chemotherapeutic effect of 5-FU • Reduced diarrhoea scores • Reduced jejunal inflammation • Increased histological score • Reduced level of PIC, IL-6 • Increased level of anti-inflammatory cytokines IL-10	[143]
Streptococcus thermophilus ST4	• Increased food intake of mice • Decreased body weight loss • Reduced mean diarrhoea score • Reduced shortening of colon length • Restored villus heights and crypts depths • Reduced level of PIC, TNF-α, IL-1β and IL-6	[97]
Bifidobacterium infantis	• Mice showed higher body weight • Mice had taller villus • Reduced level of PIC, IL-6, IL-1β and TNF-α • Down-regulated levels of T-bet (Th transcription factor) • Increased CD4+ levels • Increased CD25+ levels • Increased Foxp3+ levels • Increased Tregs levels • Reduced CD4+ IL17A+ cells	[145]
Saccharomyces boulardii	• Changed expression of Toll-like receptors, TLR2, TLR4 • Changed expression of MyD88 • Changed expression of NF-κB • Changed expression of ERK1 • Changed expression of phospho-p38 • Changed expression of phospho-JNK • Reduced level of PIC, TNF-α, IL-1β • Reduced level of chemokine CXCL-1	[165]
Saccharomyces cerevisiae UFMG A-905	• Partially reduced intestinal permeability • Reduced myeloperoxidase activity • Reduced level of chemokine CXCL-1 • Reduced mucosal inflammation • Reduced oxidative stress	[156]
Propionibacterium freudenreichii	• Reduced level of PIC, IL-12, IL-17a, IL-8, TNF- α • Changed expression of cld1	[155]

Probiotic	Effects of probiotic on ameliorating intestinal mucositis	Reference
Wild strain of *Propionibacterium freudenreichii*	• reduced weight loss • reduced gut inflammation • increased histopathological scores • induced production of Th17 cells • reduced level of IgA	[155]
Rhodotorula mucilaginosa UFMGCB 18377	• Ameliorated the decrease in food consumption • Reduced weight loss • Reduced shortening of intestinal length • Restored villus to crypt ratio • Reduced intestinal permeability • Reduced goblet cells loss • Reduced enterobacteria in intestine • Reduced MPO and EPO levels / Reduced neutrophils and eosinophils infiltration	[136]
Probiotic mixture : • *Bifidobacterium breve* DM8310 • *Lactobacillus acidophilus* DM8 302 • *Lactobacillus casei* DM8121 • *Streptococcus thermophilus* DM 8309	• Reduced level of PIC • Reduced infiltration of neutrophils • Reduced intestinal permeability • Changes of Toll-like receptors, TLR2, TLR4 signalling pathway	[158]

4. Discussion and Future Direction

Despite extensive evidence shown in this review, there is no solid guideline for treating intestinal mucositis due to chemotherapy. Current medications or other ways to combat chemotherapy-induced side effects are generally not completely effective, mostly fail to address possible long-term impacts, and may even cause additional side effects that merely exacerbate patients' sufferings[13]. Some of the interventions being used currently are limited to ice, analgesics, barrier protectors and topical antimicrobials. Hence, it is necessary to look for other possible alternatives to treat IM[136]. Probiotics have clearly shown to be a valuable adjuvant therapy in treating intestinal mucositis. Many strains successfully reduce levels of PIC, inhibit inflammatory pathways, and strengthen the gut's physical defence system. Probiotics also manage to prevent adherence of pathogenic bacteria and prevent gut cell apoptosis.

When patients undergo cancer treatment, they are at risk of gut dysbiosis. They will be less protected from harmful external factors due to lack of commensal GM and also due to a weak immune system from cancer itself as well as the chemotherapy drug.

Supplementing with probiotics before, during and after chemotherapy can increase one more level of protection for cancer patients. Further research is required to investigate the integration of probiotic interventions into the clinical management of IM, effectively halting mucositis in its track. With the current understanding that the gut microbiome plays a crucial role in all stages of intestinal mucositis, including initiation, upregulation, message generation, amplification, signaling, ulceration, and healing, it is anticipated that this integration will be realised in the near future. There is also a risk of bacterial translocation, causing invasive infections. Immunocompromised cancer patients would be particularly vulnerable, especially the risk of bacteraemia or sepsis, worsening patients' survival and mortality rate. However, with all of these studies mentioned, there were no events of bacteraemia or sepsis. Probiotics are beneficial. They inhibit the invasion of pathogens and strengthen the immune system. It is also important to note that probiotics do not compromise 5-FU efficacy, supporting their further use[17].

As shown in this review, there is a large shortage of studies conducted on humans. Although some of the preclinical studies show promising results, they might not translate similarly to humans. Most of the studies are also done in mice induced for mucositis or induced with carcinoma, which could be different from humans with chemotherapy-induced mucositis with underlying CRC. Perhaps this is why there is currently no definitive proof supporting the use of probiotics in treating IM. Therefore, this review calls for future study of ethical, large, randomised, double-blind human trials. This is to further assess the capability of probiotics in real-life clinical settings.

Studies should include isolated strains that can ameliorate all side effects of IM rather than just ameliorating one specific side effect. Other than that, the most suitable doses along with the most effective probiotic strains or combination of different probiotic strains, should also be further evaluated to maximise the beneficial effects of probiotics on the host. Also, human factors such as genetics[184,185], race, and environmental factors could affect patients' response to probiotics supplementation. Thus, knowing the correlation between these external factors and response to probiotics should be studied as well. By doing this, we might be able to predict which patients are specifically at risk of developing certain side effects. Perhaps we can tailor the probiotic supplementation to patients' needs, thus developing a personalised probiotic regimen for each patient to reach optimal treatment outcomes. Individual variability is impossible to predict; however, more studies on the association of certain external factors or internal factors, such as gene polymorphism, can be a starting point for having proper guidelines for IM therapy.

A challenge in using probiotics as a therapeutic method would be modulating its effects to target inflamed tissues while leaving normal tissues undisturbed and, most importantly, without disturbing the efficacy of 5-FU. Evidence showed probiotics do not affect the efficacy of 5-FU and its actions on tumour cells. However, similar to other studies, probiotics' influence on 5-FU's mode of action is only primarily known in rat studies. There has yet to be a large human trial to allow full justification. The challenge of maximising probiotic protection of mucosa while minimising the protection of tumour will need to be overcome. Perhaps, new techniques or technology should be used to overcome this challenge.

Also, probiotics are required to remain in the body for a considerable amount of time. So far, it is uncertain how long the effects of probiotics will last and whether the effects will continue to last even after probiotics are no longer in the body[186]. This review also does not discuss the viability of probiotics after going through the digestive tract, such as stomach acid and digestive enzymes[146].

5. Conclusion

In conclusion, this review shows promising evidence that would encourage future studies on the use of probiotics in ameliorating the side effects of 5-FU or chemotherapy in general. More studies are important, as we have seen that IM is a complex process, and its association with gut microbes is even more complex. 5-FU continues to be used today, and yet there is only a superficial understanding of the relationship between GM and 5-FU. This review highlights that GM plays an important role in the host's response to 5-FU. Also, this review gives a framework on how probiotics may increase the efficacy and lessen the side effects of 5-FU; thus, future studies on this will be beneficial to many patients that use 5-FU. There has yet to be fully validated that probiotics could be the magic bullet, and for now, it serves better as an adjuvant targeted therapy. Nevertheless, this emerging field of research provides hope for finding safer and more effective treatment options for CRC and mitigating the adverse effects associated with traditional chemotherapy. These approaches not only combat cancer but also preserve patients' quality of life during treatment. Continued efforts in this direction may open new avenues for personalized and targeted approaches to CRC treatment, revolutionizing the way we combat this devastating disease.

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