

# The Anti-neoplastic Effects of Probiotics and Prebiotics against Colorectal Cancer: A Systematic Review

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Received: February 25, 2022

Accepted: April 15, 2022

Online Published: April 20, 2022

doi:10.5539/jfr.v11n2p35

URL: <https://doi.org/10.5539/jfr.v11n2p35>

## Abstract

With the world's incidence of non-communicable diseases (NCDs) increasing, colon and rectal cancers now form the 3<sup>rd</sup> most common form of cancer globally, the need to find new solutions to colorectal cancer (CRC) is paramount, as current treatment is limited and comes with many unfavourable side effects. Studies on probiotic bacteria and prebiotic compounds spanning the last ten years reveal promising results describing their ability to act against colorectal cancer development. After screening papers with a specific inclusion criterion, 23 papers were selected for this review. The primary endpoints, biomarkers, and other data were analysed. The results show that overall, the prebiotics and probiotic bacteria included in this study (predominantly the genera *Lactobacillus* and *Bifidobacterium*) have promising anti-neoplastic effects against colorectal cancer, although in varying amounts. Other prebiotics such as fructooligosaccharides, branched fructans, and other plant extracts, were shown to have equally positive effects. The concept of using probiotics/prebiotics in addition to established cancer treatment seems more feasible with the various benefits highlighted in this review. At the very least, probiotics/prebiotics may be useful adjuvants, to be used alongside pre-existing colorectal cancer treatment. Probiotics/prebiotics may help alleviate some undesirable side effects of pre-existing treatment (i.e., fluorouracil) such as dysbiosis. Thus, this review aims to build upon the foundations established in microbiome research and encourage the course of future prebiotic and probiotic testing, to further our understanding related to the effect of probiotics/prebiotics on gut health and help treat the growing burden of colorectal cancer.

**Keywords:** Adjuvant, *Bifidobacterium*, colorectal cancer, dysbiosis, *Lactobacillus acidophilus*, mechanism, prebiotic, probiotic, public health, systematic review

## 1. Introduction

### 1.1 Background

Colorectal cancer has been an increasing concern in many developed and developing countries. For instance, in the United States, colorectal cancer is the 3<sup>rd</sup> most prevalent form of cancer, but CRC (colorectal cancer) incidence is gradually increasing globally (DeBarros and Steele, 2013). The 'nutrition transition concept' may help explain the increasing rates of NCDs experienced by many low-middle income countries, as well as explain increasing colorectal cancer prevalence (Popkin, 2006). This is likely due to the increasing availability of ultra-processed foods, aka 'western diets,' including large amounts of red meat, processed foods and relatively small amounts of fibre, fruit and vegetables (Kasdagly et al., 2014). More and more researchers are turning to nutrition-based solutions to lower the burden of NCDs such as colorectal cancer, as better nutrition can help prevent the onset of various diseases. The need for better nutrition intervention has even been recognised as the second sustainable development goal from the 2015 United Nations sustainable development summit.

With diet emphasised as a massive contributor to the onset of CRC, diet-based solutions that affect the microbiome have been studied increasingly by researchers in the last 10 years. Diet is hypothesised to modulate the human gut microbiome, and so logically investigating the consumption of prebiotics and probiotics sparked interest as a diet based solution to improve the onset and development of CRC (Ambalam et al., 2016). It is established that the microbiome is involved in so many different facets of our health, such as modulating immunity: the crosstalk between the microbiota and the immune system allows the host to recognise and tolerate oral food antigens and also initiate an immune response to pathogens and other harmful microbes

(Gopalakrishnan et al., 2018). The last 10 years of research have also influenced our understanding of the reverse; the microbiome's effects on colorectal cancer, and other types of cancers. Currently, colorectal cancer therapy is relatively limited, with options of either surgery, radiotherapy, chemotherapy, targeted therapy, or immunotherapy. The side effects of these therapies can be unfavourable and often cause other complications. Therein lies a huge area of research still to be conducted for improving cancer therapy efficacy and reducing unfavourable side effects. Whilst cancer research at large still has a long way to go, probiotics and prebiotics have been shown to have promising anti-neoplastic benefits, and they are on their way to establishing themselves for cancer prevention, adjuvant therapy and possibly new cancer treatment.

This review provides a detailed analysis of the relevant studies in the literature, with the extracted datasets in three consecutive summary tables (see tables 4, 5 and 6). This will enable us to identify the evidence supporting the use of probiotics and prebiotics against CRC, whilst also looking at each studies' methodology to check the reliability of the evidence.

### 1.2 Pathogenesis

Colorectal cancer is hypothesised to be influenced by gene mutations, epigenetic alterations, and local inflammatory changes, of which many epigenetic alterations have been identified over the last 30 years (Grady and Markowitz, 2015). Genomic and epigenetic alterations causing CRC include issues with chromosomal instability, microsatellite instability, and other mechanisms. Colorectal cancer occurs because the epithelial cell compartments (crypts) house lesions that progress into adenomatous polyps. At this stage, these polyps are non-cancerous growths but with time, can grow into colon walls, and will eventually lead to metastatic cancer (Kruzelock and Short, 2007). Colorectal cancer can be classified into 4 distinct stages, as listed in table 1.

Table 1. Table 1 shows the four stages of cancer described by the level of invasion

Stage I/A	Stage II/B	Stage III/C	Stage IV/D
The cancer cells are confined to the innermost cell wall.	Tumour penetrates muscularis/adjacent organs or other structures but has not spread to the lymph nodes yet.	Cancer cells have spread to the lymph nodes, but the cancer cells have not yet spread to other parts of the body.	The cancer cells have invaded the lymph nodes and have spread to distant parts of the body.

### 1.3 Assessment Scales of Colorectal Cancer

The main prognostic biomarker used is carcinoembryonic antigen (CEA) which is expressed in colorectal malignancies (Alves Martins et al., 2019). Higher levels are associated with CRC progression, but high levels can also indicate other diseases like Inflammatory bowel disease, other cancers, liver disease and pancreatitis.

Colorectal cancer can be broadly categorized into three types: sporadic, inherited, or familial. 75% of CRC cases are sporadic, which is the type influenced by diet and lifestyle, and so logically can be positively influenced by diet, by using dietary interventions such as probiotics and prebiotics.

### 1.4 Microbiota

The human gut microbiome houses trillions of microbes that interact with each other, and these microbes play a critical role in modulating overall human health. The effects of the microbiota are mediated by the metabolites that are produced such as short-chain fatty acids (SCFAs). These activate G protein-coupled receptors which in turn promote various effects such as regulating immune response and inflammation, providing energy, promoting glucose homeostasis, increasing tumour suppression and hormonal regulation (Mohajeri et al., 2018). Pathogenic bacteria like *Fusobacterium* and enterotoxigenic *Bacteroides fragilis* may enter the microbiome, these, and other bacteria are associated with increased tumorigenesis. Part of colorectal cancer progression can be attributed to a dysbiosis of the gut microbiome, where the ratio of normal commensal, harmless bacteria and pathogenic bacteria are imbalanced. This can occur due to many different reasons: i.e., drugs, pathogens, but most notably diet (and we see that diet is the number one cause of colorectal cancer). But interestingly current drug treatments for CRC cause gut dysbiosis as a side effect (Ichim, Kesari and Shafer, 2018), which has further encouraged scientists to look at probiotics and prebiotics as a possible adjuvant to cancer therapy in that they might resolve the gut dysbiosis as well as perform its anti-neoplastic effects.

### 1.5 Probiotics

Probiotics may help alleviate colorectal cancer through a variety of proposed mechanisms. Firstly, by modulating immune response and reducing pro-inflammatory cytokines, like TNF- $\alpha$ , IL-6, IL-10, IL-17. Secondly, by

multiplying and colonizing the gut environment thereby inhibiting the colonization of harmful pathogenic bacteria. Thirdly by transforming the epithelial wall lining (upregulating tight junction protein expression and increasing the production of mucin). Lastly by promoting apoptosis in cancer cells (Perillo et al., 2020). Many varieties of probiotic bacteria have been shown to have positive anti-neoplastic effects on colon cancer within the literature. Lactic acid bacteria, specifically the genus *Lactobacillus*, is seemingly effective, but also probiotic bacteria of the genera *Bifidobacterium* and *Enterococcus* have been shown to have promising anti-neoplastic effects. The probiotic bacteria work to suppress inflammatory cytokine expression, modulate gene expression, modulate T cell-specific responses, and increase SCFA production (which has been shown to inhibit the growth of cancerous human cell lines many times in the literature). Furthermore, other antiproliferative effects of probiotics may be attributed to the inactivation of mutagens or carcinogens and the lowering of intestinal pH (Raman et al., 2013).

Commonly used prebiotics include Inulin, fructans, fructooligosaccharides, galacto-oligosaccharides, and other oligosaccharides, which have been proven to confer health benefits against colorectal cancer primarily by increasing the growth of beneficial gut bacteria and thus promoting most of the health benefits of probiotics (i.e., increasing SCFA production, altering pH, modulating immune response etc).

### 1.6 Rationale and Justification

Gut microbiome and cancer research has been an emerging area of interest since the mid to late 2000s, but in the last 5 years alone, there has been a surge in interest, with thousands of studies being published. This review will look at a select few papers, published since 2010, and assess from the exhaustive summary of evidence, whether the use of probiotics and prebiotics provide benefit against colorectal cancer development. The review will utilise a critical appraisal system to assess the validity of the data of each paper, then look at the hypothesised mechanisms to explain the effects.

This is significant as the literature on the microbiome and its effects on the host are still relatively new, especially the literature on the impact of modulating the microbiome against cancer. The rationale behind producing this review is that, with the vast amount of available literature, there is a need to separate the robust, predictable, and reliable data from the other literature. Reviews like this help navigate the progression of research and define areas of interest for future studies and help us draw conclusive insights (i.e., are certain tests that work well in animals also suitable for humans?). More studies and discoveries grow the already expanding list of beneficial probiotics/prebiotics from which researchers may select, for use in future studies. This review works towards completing the aims through the objective measures listed in section 2; the process is outlined in section 3.

This review aims to examine whether the findings in each paper are consistent with each other and to refine the quality data sources from the vast excess of published articles. Again, this review takes a relatively small sample of 23 papers from the vast literature pool in microbiome research, and with these papers, evaluates, refines, and analyses the evidence. This will enable us to extrapolate our findings and answer the hypothesised question: Is there enough evidence to suggest that probiotics and prebiotics have anti-neoplastic effects against colorectal cancer?

## 2. Aims and Objectives

This review aims to extrapolate from the evidence and conclude, whether probiotic bacteria and prebiotic compounds are effective against colorectal cancer.

The objective measures include examining primary data sets that are one of three types: papers that look at colorectal cancer in humans, papers that look at models of colorectal cancer cell lines *in vitro*, and papers that look at colorectal cancer in animal models. This was carried out by:

- Extracting data from studies and appraising the quality of the studies' methods to determine the reliability of the data.
- Identifying data in each study and comparing for common themes and trends observed in the literature.
- Extrapolating data to identify the potential therapeutic benefits of probiotics and prebiotics on real patients, which holds promise to potentially work alongside, or in lieu of, existing colorectal cancer treatment.

These objective measures, outline how this review intends to answer the hypothesis, that if prebiotic/probiotic compounds have been shown to have anti-neoplastic effects via the proposed mechanisms, then the use of these compounds may be beneficial in colorectal cancer patients

### 3. Methods

The question of finding the anti-neoplastic effects of probiotics and prebiotics against colorectal cancer involves an in-depth literature review, which came to fruition after creating the research question, using the PICO (population, intervention, comparison, outcome) search strategy. We then conducted a structured MeSH (medical subject headings) PubMed Search, and a generic search using the University of Westminster's library to find the papers. After screening via specific inclusion criteria, the papers were then appraised to identify the validity of the evidence, and the robustness of the studies' experiments. Then the papers were pooled together in a summative data extraction table (sections 4.2, 4.3, 4.4). The findings from each study are discussed and cross-compared to finalise an overall opinion of this area of the literature. The significance of the presented evidence is discussed relating to the wider task of lowering the global prevalence of colorectal cancer and possibly other NCDs.

#### 3.1 Search Strategy

A MeSH search using terms drawn from the PICO search strategy was conducted in the PubMed database, and then a search using key terms from the PICO table was completed in the Westminster University Library database. The PubMed MeSH search involved 4 main concepts: probiotics, prebiotics, colorectal cancer, and neoplastic effects, as outlined in table 2. The Westminster library search used the phrase 'anti-neoplastic effects of probiotics and prebiotics against colorectal cancer,' revealing 156 papers that were then screened for relevance.

Table 2. Table 2 shows the exact MeSH terms used to search the PubMed database

Concept #1: probiotics →
"Probiotics"[Mesh] OR bacteria*[tw] OR yeast*[tw] "live bacteria*" [tw] OR "live yeast*" [tw]
Concept #2: prebiotics →
"Prebiotics"[Mesh] OR "whole food*" [tw]
Concept #3: colorectal cancer →
"Colorectal Neoplasms"[Mesh] OR "colorectal cancer" [tw] OR "colon cancer" [tw]
Concepts #4: antineoplastic effects →
"Anticarcinogenic Agents"[Mesh] OR "Antineoplastic Agents"[Mesh] OR "anti cancerous" [tw] OR "anti cancer" [tw] OR "cancer therapy" [tw] OR "anticarcinogenic" [tw]

#### 3.2 PICO & Prisma Flow Chart

The PICO format was used firstly to define the PICO question, and then to help plan our search strategy. Table 3 shows the PICO search elements with their related keywords/phrases to aid the literature search.

Table 3. Table 3 displays each PICO element

PICO ELEMENT	KEYWORDS/PHRASES
P (Population)	Patients or animals with Colorectal cancer or induced cancer of the colon (i.e., artificially with DSS etc).
I (Intervention)	Probiotic/prebiotic treatment.
C (Comparison)	No intervention, placebo, or treatment with other probiotics.
O (Outcome)	Reduction in postoperative complications, reduction in the level of pro-inflammatory cytokines, increase in short-chain fatty acids, decrease in intestinal pH, anti-proliferative effects on cancer cell lines, and fewer adenomatous polyps.

The intervention was defined as an experiment that involved administering any type of probiotic bacteria or popular prebiotic compound. The comparisons would be unique to each study but broadly consists of untreated controls, a cancer-induced mouse model without probiotic/prebiotic intervention, or treatment with other prebiotics/probiotics. Most of the included studies had various tests, but we looked for papers whose primary outcome measures determined the effect on immune response, SCFA production, tumour proliferation/overall tumour load, and changes in microbial gut diversity. The population was kept relatively broad, to include studies that included humans, mice/rats, and in vitro studies as the area of literature is still relatively new. The literature search aimed to find papers that fit the specific criteria and would provide evidence to support the use of probiotics and prebiotics, and the PICO search helped define a search strategy for this. The steps taken throughout the literature search are outlined below (fig 1).

### 3.3 Inclusion Criteria

The data screening process in PubMed using the MeSH themes (1,2,3 and 4 listed above) produced large amounts of papers but combining them revealed only 20 relevant papers by title. An additional generic search using the terms ‘Probiotics AND Prebiotics AND colorectal cancer’ revealed 32 papers relevant by title, leaving 54 papers in total from PubMed. The Westminister search brought up 156 papers, but not all the papers were suitable for the study. They were filtered out during the screening process. From both database searches, articles were excluded if the articles did not present primary research data (i.e., reviews) if the papers were published before 2010 (setting the date window from 2010 to 2021) if the papers did not focus on probiotic bacteria or compounds considered as prebiotics, if the papers did not specifically address colorectal cancer, and if the papers were not available in English. Papers that included the use of compounds that were not prebiotics but were some other plant derivative or food product were excluded.

The remaining 23 papers were split into categories of *in vitro* studies, including different models (i.e., the simulator of human intestinal microbial ecosystem, or ‘SHIME’ model), *in vivo* animal studies (i.e., transgenic mouse models, colon cancer graft models etc), and human studies. We decided to include studies that were *in vitro*, *in vivo* mice/rat studies and human studies, because the evidence within the literature is still relatively limited, and we found common themes throughout all the studies. In this manner, we could see how different probiotics/prebiotics were used in the *in vitro* stage, and if these were successful in the animal testing and human studies, which reinforces the evidence supporting the anti-neoplastic effects seen in the existing literature.

### 3.4 Data Extraction

Data selected from the studies included: the employed methods, the primary endpoint and how this was measured, the probiotics/prebiotics of interest, the treatment duration, and other details. The tables (see tables 4, 5 and 6) created a summary of our findings that allow us to see the relationship between the studies in this area of the literature, thus allowing us to assess the strength of the evidence in this review.

### 3.5 Quantitative Study Appraisal

The studies included in this review have undergone a modified version of the quality assessment tool of quantitative literature outlined by Thomas, et al (2004), which looks at selection bias, study design, data collection methods and overall intervention integrity.

## Prisma Flow Chart

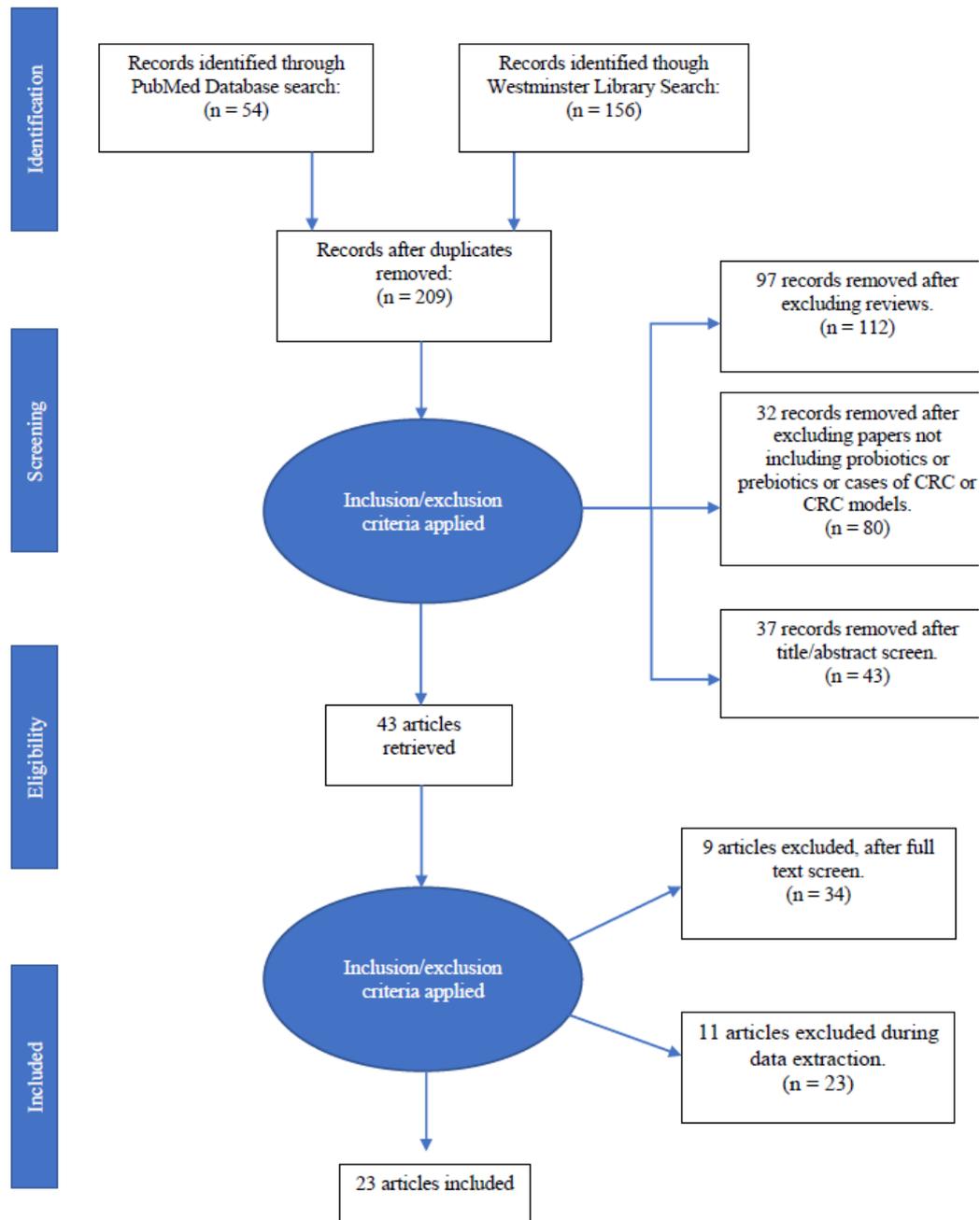


Figure 1. Figure 1 displays the process of identifying and screening the papers to decide which papers to include in our dataset

## 4. Results

### 4.1 Findings

Throughout the reviewed studies, there were common recurring probiotic bacteria that reported positive effects, these were the *Lactobacillus* and *Bifidobacterium* genera. We also reported that the mechanisms in which the probiotics mediated positive anti-neoplastic responses, were through mediating pro-inflammatory markers (an immunomodulatory response), through increasing the production of beneficial SCFAs, through changing the microbial flora (namely reducing pathogenic bacteria associated with colorectal cancer, such as *Bacteroidetes*) and through initiating apoptosis (although the mechanism for this process is still not clear). The review also includes studies with novel data, i.e., new prebiotic compounds and probiotic bacteria that reported beneficial

anti-cancerous effects, which were not previously established in the literature, and newly proposed mechanisms to help explain the antiproliferative effects and more.

Themes that related to the positive anti-neoplastic effects of probiotics and prebiotics were identified, these are detailed below and discussed in more depth in section 5.

Theme 1: increases in SCFA production. The marked increased SCFA that were the most reported were: butyrate, acetate, and propionate.

Theme 2: changing microbial composition. Studies reported that the 4 dominant phyla observed in the gut microbiome changed: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*. The ratio between *Bacteroidetes* and *Firmicutes* is regarded as significant (Magne *et al.*, 2020) and relative to the dysbiosis of the gut, i.e., an imbalance of this ratio is often observed in inflammation. Probiotics and prebiotics have been shown to reverse the dysbiosis and restore this ratio and reduce the amount of harmful CRC associated bacteria, such as *Bacteroides fragilis*.

Theme 3: modulating inflammatory response. Probiotics in the studies reduced pro-inflammatory cytokine markers such as interleukin 8 (IL-8), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), and tumour necrosis factor alpha (TNF- $\alpha$ ). They also increased anti-inflammatory cytokine markers, i.e., IL-4, IL-2, IL-6, and signal transducer and activator of transcription 3 (STAT3).

Theme 4: increasing T-cell expression. Many of the human studies include reports of increasing expression of CD8+ cells (cytotoxic cells that induce apoptosis) thus restoring the CD4+/CD8+ ratio, CD26L, changing the expression of natural killer (NK) CD3-CD49b+ cells and changing the dendritic phenotype of CD83-123, CD83-HLADR, and CD83-11c.

Theme 5: inducing apoptosis. Studies report that probiotics that increase SCFAs also induce apoptosis because the SCFAs activate caspase-3 and caspase-7, which are key effectors of apoptosis. The JNK (c-Jun N-terminal kinase) mediated apoptosis mechanism was also explored in one study, whereby ferrichrome was found to be the molecule initiating the JNK signalling pathway and induced apoptosis in cancerous cell lines.

Theme 6: decreased tumour load. The animal studies particularly, have reported a significant decrease in the tumour diameter and tumour number in cancer-induced mice models. This is a result of decreased proliferation rate and increased apoptosis (described in theme 6) in the cancerous cells. There was also a reported decrease in polyp formation.

Theme 7: enzyme activity. The level of key effector enzyme activity was tested for in the studies. Caspase-3, caspase-7,  $\beta$ -glucuronidase ( $\beta$ -GA), and glutathione-S-transferase (GST) all have their specific roles in cancer progression and so act as tumour progression checkpoints. Studies show that prebiotics and probiotics were able to positively affect the level of these enzymes.

Theme 8: cytotoxicity and genotoxicity. One of the main primary endpoints for studies included in this review was the effect of probiotics/prebiotics on faecal water genotoxicity and cytotoxicity.

Theme 9: E-cadherin/N-cadherin ratio. Studies were able to show that probiotics/prebiotics were able to reverse the 'Cadherin switch,' a phenomenon where cancer patients often experience a downregulation in E-cadherins and an upregulation of N-cadherins. The studies showed that certain probiotics were able to restore the correct ratio of these cadherins.

The studies revealed that certain probiotic bacteria exerted positive anti-neoplastic benefits more commonly but were also studied more, so more data on their effects were available. These most common types of probiotic bacteria are listed as follows. *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Bifidobacterium lactis*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium breve*, *Streptococcus thermophilus*, and *Streptococcus thermophilus*.

The probiotic bacteria that are less frequently reported, but still demonstrate positive benefits are as follows: *Saccharomyces boulardii*, *Lactobacillus johnsonii*, *Lactobacillus bulgaricus*, *Lactobacillus delbrueckii*, *Bifidobacterium pseudocatenulatum*, *Lactobacillus buchneri*, *Lactobacillus farciminis*, *Lactobacillus helveticus*, *Lactobacillus salivarius*, *Lactobacillus paracasei*, *Pediococcus acidilactici*, *Enterococcus faecalis*.

Prebiotic compounds included in the studies that exert positive benefits are as follows. Branched fructans from *Agave angustifolia*, green cincau from *Premna oblongifolia* Merr, corn tortilla (red, blue, white, and yellow varieties), gypenoside Rb3, gypenoside Rd, agar-derived sugars, glucooligosaccharides (oligoalternan, oligodextran), Galactooligosaccharides containing  $\beta$ -1,6 and  $\beta$ -1,3 linkages, saponins extracted from

*Gynostemma pentaphyllum* (GpS) *Ganoderma lucidum* (GLP), ferrichrome, *Agaricus blazei* Murill (AbM) from Basidiomycetes mushrooms, beans, and Arabinoxylan-oligosaccharide from wheat bran extract.

#### 4.2 Human Studies

Table 4. Intestinal microbiota modifications from probiotics/ prebiotics in human studies

Study	Subject Details	Study design & Intervention	Primary end points/ Techniques used	Samples	Results	Appraisal, Commentary/Criticism and Remarks
Kotzampasi, et al.	164 participants (115 men, 49 females) with varying stages of colorectal cancer, all in need of a low anterior resection, Recto-sigmoidectomy, Right hemicolectomy or total colectomy.	4 capsules of probiotic or placebo were given the day before surgery. On the day of, and 14 consecutive days after surgery, 2 capsules a day were given. (1 capsule = 4 different strains of Probiotic at $5.5 \times 10^9$ cfu (colony forming unit)).  *The probiotic capsule contained: <i>Lactobacillus acidophilus</i> LA-5 $1.75 \times 10^9$ cfu, <i>Lactobacillus plantarum</i> $0.5 \times 10^9$ cfu, <i>Bifidobacterium lactis</i> BB-12 $1.74 \times 10^9$ cfu, <i>Saccharomyces boulardii</i> $1.5 \times 10^9$ cfu	The Physiological and Operative Severity Score for the end-Points of Mortality and morbidity (POSSUM) scores were used to measure postoperative complications within 30-day post-op.  Real-time polymerase chain reaction (PCR) measured levels of inflammatory markers TNF and IL-6, and gene expression of <i>SOCS3</i> .	Subjects account for postoperative complications. Blood samples.	The prevalence of postoperative complications in the probiotic group was 28.6% compared to the placebo group (48.8%). Infectious species <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , and <i>Staphylococcus aureus</i> were found to be lower in the probiotic group compared to the placebo group in all accounts. The probiotics group were discharged on average 2 days earlier than their placebo counterparts (8 days, 10 days respectively). The probiotics group reported a clearer correlation of <i>SOCS3</i> control of TNF and IL-6, indicating that <i>SOCS3</i> levels increased as IL-6 and TNF increased to control the overwhelming cytokine response ( <i>SOCS3</i> is known to control overwhelming cytokine responses), thus exerting better modulation and better control of the cytokines.	This paper scored strongly across all components of the quality assessment tool. The paper scored as 'strong' when assessed against selection bias, study design, confounders, blinding, data collection methods, withdrawals and drop-outs, intervention integrity, and analysis. However, the relative success of the study can be viewed because of the premature ending of the study. No reports of a clear correlation between the probiotics group showing higher levels of cytokine suppressing gene <i>SOCS3</i> , only a better correlation.
Zaharuddin, et al.	52 participants with colorectal	Capsules were consumed twice daily for 4 weeks	Enzyme-linked	Blood samples	There was a significant	This study scored highly in the quality

<p>cancer. (24 male, 18 female). Participants included were from varying stages of cancer: stages I-III.</p>	<p>post-surgery. Capsules were consumed twice daily for up to 6 months. 3.0x10<sup>10</sup> cfu, of six probiotic strains, in a 107mg capsule. Containing <i>Lactobacillus acidophilus</i> BCMC 12130, <i>Lactobacillus lactis</i> BCMC 12451, <i>Lactobacillus casei subsp</i> BCMC 12313, <i>Bifidobacterium longum</i> BCMC 02120, <i>Bifidobacterium bifidum</i> BCMC 02290, <i>Bifidobacterium infantis</i> BCMC 02129.</p>	<p>immunoassay (ELISA) is used to measure the proinflammatory cytokines TNF-<math>\alpha</math>, IL-6, IL-10, IL-12, IL-17A, IL-17C, IL-22, and interferon gamma (IFN-<math>\gamma</math>).</p>	<p>before and after the intervention .</p>	<p>reduction in the level of TNF-<math>\alpha</math> IL-10, IL-12, IL-17A, IL-17C, IL-22, IL-6 pro-inflammatory markers in the probiotics group compared with their baseline preoperative levels. However, IFN-<math>\gamma</math> did not decrease, the level was about the same pre-operation and post-op with the probiotic. The probiotic and the placebo group post-treatment showed a lower level of TNF-<math>\alpha</math>, IL-10, IL-12, IL-17A, IL-17C, and IL-22 and interestingly for IL-6, the placebo group showed lower levels. For IFN-<math>\gamma</math>, the levels in both groups were about the same.</p>	<p>appraisal tool, its double-blind study design was explained fully, and its data collection methods and intervention integrity were strong. However, 15 participants withdrew from the study and 8 patients did not continue due to compliance issues and lack of follow up. But a strong quality score. The exact role of TNF-<math>\alpha</math> in cancer promotion is not fully understood, so its significance may not be fully clear. The placebo group also showed a reduction in IL-6, which was not explained.</p>
<p>Gianotti, et al. 31 patients undergoing colorectal resection.</p>	<p>A dose of 2.0x10<sup>7</sup> cfu/d or a stronger dose at 2.0x10<sup>9</sup> cfu/d mixture of <i>Bifidobacterium longum</i> (BB536) and <i>Lactobacillus johnsonii</i> (La1) at a ratio of 1:1, or a placebo of 2 doses were given orally 3 days before the operation, and on days 2 to day 4 after the operation for a total of 6 treatment days.</p>	<p>Real-time quantitative reverse transcription PCR (Real-time qRT-PCR).</p>	<p>Stool samples were collected before treatment, during surgery (day 0) and 5 days post-op. Colonic mucosa sample.</p>	<p>The group that took the high dose of probiotic had a high level of adherence at 60%, vs 27.2% (High dose vs low dose respectively). And 0% in the placebo group. The <i>Enterobacteriaceae</i> (30% in the high dose, 91.8% in the low group) and <i>enterococci</i> counts were lower in the high dose probiotic group. The results were similar to the <i>enterococci</i> group but did not reach statistical significance. Probiotics didn't</p>	<p>This study scored highly in all the quality assessment criteria. This study is relevant as taking probiotics before or after colorectal surgery has been shown in other studies to have positive benefits. Evidence supports that <i>La1</i> and <i>BB536</i> reduce the concentration of pathogens and modulate local immunity. Potential to reduce inflammation in colorectal resection patients.</p>

Odamaki, et al.	420 adult volunteers for epidemiological study. 32 healthy adult volunteers (11 male, 21 female) for clinical study.	The control group received 200ml of high temperature pasteurized milk or 160g of yoghurt containing $1.0 \times 10^9$ cfu of lactic acid bacteria. supplemented with <i>Bifidobacterium longum</i> BB536, <i>Lactococcus lactis</i> , <i>Streptococcus thermophilus</i> and <i>Lactobacillus delbrueckii bulgaricus</i> .	In the epidemiological study, a questionnaire about lifestyle habits was asked. qRT-PCR was used to measure the effectiveness of the probiotic on enterotoxigenic <i>Bacteroides fragilis</i> (ETBF) cell number	Faecal samples.	affect dendritic cell phenotype or activation. Dendritic phenotypes (CD83-123, CD83-HLADR and CD83-11c) were significantly less expressed in patients that were colonized with La1. The prevalence of enterotoxigenic bacteria was lower in the probiotic group, the baseline cell number of ETBF did not change upon taking the placebo in the milk group. In a follow-up study, the subjects whose ETBF level fell in the probiotics group were followed up 12 weeks later, and their ETBF levels were restored to pre-treatment levels.	This study scored highly across most of the quality assessment criteria. There was no explicit mention of blinding in the study. Evidence suggests that the use of the probiotic could potentially be useful in cases where ETBF has contributed to the development of CRC. However, the study emphasises how <i>B. longum</i> BB536 was the main factor, but the probiotic also contained <i>L. delbrueckii bulgaricus</i> , <i>S. thermophilus</i> , and <i>L. lactis</i> . The study does not tell us if the individual strains or the combination led to these results.
Frederich, et al.	16 patients (10 male, 6 female) with familial adenomatous polyyps (FAPs) and Ileal pouch-anal anastomosis (IPAA).	Sulindac monotherapy (300mg/d): 1 tablet of 100mg in the morning and 2 in the evening. Combination of VSL#3 (4 <i>Lactobacilli</i> , 3 <i>Bifidum</i> , and 1 <i>Streptococcus</i> species ( $9.0 \times 10^{11}$ bacteria/day) and inulin (plant polysaccharide) (12g/d). Combination of sulindac and VSL#3/inulin.	Cell proliferation was measured via staining with monoclonal antibody (MIB-1), detoxification capacity, gas chromatography for SCFA contents, pH metre for pH, and tetrazolium based colorimetric assay for cytotoxicity of faecal	Biopsies of the pouch. Faecal samples.	Group 1 (patients who received sulindac) had reduced cell proliferation, increased GST activity, increased cytotoxicity, decreased SCFA production, and similar faecal pH. Group 2 (patients who received VSL#3/inulin) had reduced cell proliferation, increased GST	1 patient had to stop participating in the study due to post-operative complications. The study cohort was relatively small. But otherwise, the study scored highly on most of the quality assessment criteria. Sulindac has proven to reduce the number and size of adenomas in FAP patients, so the expectation was that the combination of

			water.		activity, reduced cytotoxicity, reduced SCFAs and increased faecal pH.	these two with the probiotic formula would have positive effects, but the results were not statistically significant, furthermore, the experiment did not test the effect of VSL#3 alone on the FAP patients, which would have likely yielded a better point of comparison.
Gao, et al.	22 patients (12 male, and 10 female) with colorectal cancer similar in gender, age, body mass index (BMI), and cancer stage. An additional 11 healthy volunteers were included as the control.	3 groups, a group received perioperative placebos (CGT group) and a group received probiotics (PGT group). Each received a dose x 3/d for 5 days. The Healthy group (HGT) was used as a comparison. Each probiotic dose contained 1.0x10 <sup>7</sup> cfu/g <i>Bifidobacterium longum</i> , <i>Lactobacillus acidophilus</i> , and <i>Enterococcus faecalis</i> . The placebo group received maltodextrin.	16S rRNA PCR. Chao, ACE, Shannon and Simpson indices were used.	Tissue biopsies.	Group 3 (sulindac + VSL#3 + inulin) had increased cell proliferation, increased GST activity, increased cytotoxicity, negligible SCFA increase and decreased faecal pH. All the secondary endpoint markers (cytotoxicity, pH and SCFA) were statistically insignificant. The probiotics increased the richness and diversity of the mucosal microbes. 416,599 reads were obtained. At the genus level, there were 188 in the CGT group, 198 in the PGT and 201 in the HGT group (additional 11 volunteers). In the probiotics group, there was a significant reduction in <i>Peptostreptococcus</i> , <i>Comamonas</i> , <i>Fusobacterium</i> and expansion of <i>Enterococcus</i> and Proteobacteria.	There was no significant difference in gender, BMI, and cancer stage in the participants. This study scored highly for the selection bias criteria, and generally for all the quality assessment criteria. The study demonstrated a comprehensive detailed explanation of the microbiome's increased diversity in the probiotics group. This study was unique in this review, in that its focus was on pyrosequencing the increased diversity as the primary endpoint.
Windey, et al.	20 healthy volunteers with regular dietary patterns.	10 subjects received 2 x 5g wheat bran extract/d (WBE, containing arabinoxylan-oligosaccharides), and the other 10 received 2 x 5g maltodextrin/d. Then the WBE group swapped to placebo and vice versa during the second intervention period.	Denaturing gradient gel electrophoresis (DGGE). The primary outcome was faecal water genotoxicity. qRT-PCR was used to	Urine and Faeces samples.	The effect of WBE on faecal water cytotoxicity and genotoxicity was no different from the placebo. The WBE increased faecal (15)N-excretion. The	The intervention integrity was strong as was the selection bias, as this was a double-blind randomised cross over study. However, this study's limitations come from using faecal water to

<p>identify the changes in the microflora of the gut.</p> <p>Comet assay to measure genotoxicity of faecal water.</p>	<p>WBE group has lower urinary <i>p</i>-cresol excretion, but this was dependent on protein intake. No significant changes in faecal metabolite between the two groups. No difference in total SCFA excretion between the two groups. After intake of WBE, there was a modest increase in microbial diversity. Only one band class of organism was higher than the placebo, however, this difference was statistically significant.</p>	<p>assess the risk of CRC development, which may not be a good representation of the health of the colon and the risk of CRC development. Even still, the study still scored highly on most of the quality assessment criteria. The study yielded modest results. The study was carried out on healthy volunteers, so no indication of whether the same prebiotic would have the same effects on colorectal cancer patients.</p>
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This table depicts the seven human studies selected for this review. Four of these papers were done on studies focusing on the effect of probiotics and prebiotics in colorectal cancer patients undergoing corrective surgery. The probiotics and prebiotics have been shown to decrease levels of pro-inflammatory cytokine markers like TNF- $\alpha$ , IL-10, IL-12, IL-17A, IL-17C, IL-22, IL-6, and regulate gene expression i.e., Suppressor of cytokine signalling 3 (*SOCS3*). Studies include the participation of healthy volunteers.

### 4.3 In Vivo Animal Studies

Table 5. Intestinal microbiota modifications from probiotics/ prebiotics in animal studies

Study	Subject Details	Study design & Intervention	Primary ends point/ Technique used	Samples	Results	Appraisal, Commentary/Criticism and Remarks
Hu, et al.	Female BALB/c mice (6-8wks old).	1x10 <sup>8</sup> cfu/mouse of <i>L. plantarum</i> or <i>L. rhamnosus</i> were administered to two groups through oral gavage for 14 days before implanting CT26 cells into the mice. Bacteria were administered 1/week for 3 weeks at 1 x 10 <sup>9</sup> cfu. 100uL of phosphate-buffered saline was given to the control mice.	The size of the tumour was measured macroscopically. Flow cytometry to show t cell infiltration. Histopathology to identify necrosis. ELISA assay for IFN- $\gamma$ secretion from splenocytes.	Tumour, tumour cells. Spleen cells.	<i>L. plantarum</i> significantly inhibited tumour growth and <i>L. rhamnosus</i> did not affect tumour size. <i>L. plantarum</i> group had increased infiltration of CD4+ and CD8+ T cells, NK cells and CD3-CD49b+ (CD8+ levels dropped and NK CD3-CD49b+ levels increased in	This study was very robust showing excellent intervention integrity. All around this study scored highly in the quality assessment criteria. The study presents evidence that <i>L. plantarum</i> may initiate an immune response through activating Th cells. It enhances NK activity and associated IFN- $\gamma$ levels. And overall tumour growth in CT26 induced

					the spleen, in another test for the <i>L. plantarum</i> group, the difference was not significant in the <i>L. rhamnosus</i> group) So the CD4+/CD8+ ratio increased (associated with Th cell activation). Significant increase in IFN- $\gamma$ production in <i>L. plantarum</i> group.	BALB/c mice. <i>L. plantarum</i> appeared to promote migration of CD8+ and NK cells into tumour tissue which may explain the results. Overall, this is a very comprehensive study with lots of evidence to support its findings.
Reynoso-Camacho, et al.	Male Sprague-Dawley rats (4-5 weeks old).	Rats split into 6 groups. Group 1 (n=10) was the control, group 2 (n=18) and received a standard diet, groups 3-6 (n=16) were fed with powdered diets supplemented with white corn tortilla (WCT), yellow corn tortilla (YCT), blue corn tortilla (BCT) and red corn tortilla (RCT) respectively. After week 4 of feeding the animals in groups, 2-6 were injected with 1,2-dimethylhydrazine (DMH) once a week for 8 weeks.	Inspected for macroscopic lesions. H&E staining for pH and $\beta$ -GA analyses. Bicinchoninic acid protein assay was used to measure $\beta$ -GA, GST and NAD(P)H:quinone oxidoreductase 1 (NQO1). Western blot to examine the relative amounts of K-ras, $\beta$ -catenin, and $\beta$ -actin.	Rat colon sample. Cecum sample.	The corn tortilla diets reduced the incidence of adenocarcinomas. The WCT (white corn tortilla) and BCT (blue corn tortilla) group developed 77.5% fewer tumours whereas the YCT and RCT developed 55% fewer adenocarcinomas. Higher pH values were seen in the tortilla groups, $\beta$ -GA was significantly higher in the DMH group compared to the control but was lower in the ceca of rats from the BCT, WCT, and YCT groups. Tumours from the tortilla treated groups have not significantly lower expressions of K-Ras, and $\beta$ -catenin, especially in the WCT and BCT groups.	This controlled clinical trial scored well overall with each quality assessment component. The study design was appropriate. The lower $\beta$ -GA activity in the tortilla treated groups indicates lowered CRC activity because $\beta$ -GA is heightened in CRC activity. The results indicate that the consumption of corn tortillas especially WCT and BCT reduced colon carcinogenesis by a mechanism related to inhibiting cecal $\beta$ -GA, inducing GST and NQO1, and modulating K-ras and $\beta$ -catenin. It is established in the literature that K-ras and the $\beta$ -catenin pathway are related to probiotic bacteria as are $\beta$ -GA, GST and NQO1 activity, although not discussed in the paper. The significance of this study to our review is that the corn tortilla acts as a prebiotic which induces all these

Huang, et al.	Apc <sup>Min/+</sup> mice (6 weeks old).	The mice were given one of two purified saponin compounds from <i>Gynostemma pentaphyllum</i> , gypenoside (Rb3) and gypenoside (Rb), every week for 8 weeks, until the mice were 14 weeks old.	Endpoints: measure the downregulation of oncogenic signalling molecules: iNOS (inducible nitric oxide synthase), and STAT3/pSTAT3, Src/pSrc (Proto-oncogene tyrosine-protein kinase). The gut epithelium goblet and Paneth cell population through staining with hematoxylin and eosin (H&E), alcian blue and lysozyme staining. The E-cadherin and N-cadherin expression. These tests were done with qRT-PCR, and western blot analysis.	Faecal samples were taken at weeks 0, and week 8. Gut epithelium sample.	The WCT and the BCT groups. WCT and the other groups to a varying degree increased NQO1(3) levels. There was a substantial increase in goblet cells in the small intestine and colon in the treated mice and an increase in Paneth cells in the small intestine of treated mice. The treated mice showed upregulation of E-cadherin and downregulation of N-cadherin. P-STAT3 expression was reduced in colonic mucosa. P-Src and iNOS proteins were suppressed in treated mice also. Rd profoundly decreased the level of proinflammatory cytokines compared to Rb3. Rb3/Rd increased the gut microbiome's bacterial diversity, increasing the growth of beneficial bacteria such as <i>Lactobacillus</i> , <i>Bifidobacterium</i> , <i>Ruminococcus</i> , <i>Prevotella</i> and <i>Blautia</i> . Rb3/Rd also downregulated cachexia associated bacteria,	positive effects, although this angle is not explicitly covered in the paper. This study contained many different tests to describe how Rb3/Rd extracts have beneficial effects against colorectal cancer. The study design, methodology and intervention integrity were very robust. This study scored highly on most of the quality assessment criteria.
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Zhuo, et al.	Male BALB/c mice (6-8 weeks old).	Mice, except for negative controls, were induced with azoxymethane (AOM) and dextran sulfate sodium (DSS). These mice were randomly assigned to either the low dose group (lysate of 5x10 <sup>6</sup> cfu <i>L. acidophilus</i> ), high dose group (2x10 <sup>7</sup> cfu <i>L. acidophilus</i> ) or a PBS control. Mice took the lysate dose every other day for 5 weeks.	Mice colon's stained with H&E. qRT-PCR to measure cytokine mRNA levels in peritoneal macrophages and mesenteric lymph nodes. Flow cytometry to measure lymphocytes in mesenteric lymph nodes. qPCR for the microbial bacterial community. qRT-PCR to measure mRNA of TNF- $\alpha$ , and IL-10. Flow cytometry to measure M2 macrophage cells (CD206+) in the in vitro tests.	Colon tissue. Faeces.	especially <i>Butyrivimonas</i> , <i>Campylobacter</i> , <i>Dysgonomonas</i> , <i>Fusobacterium</i> and <i>Helicobacter</i> . The high dose of the <i>L. acidophilus</i> lysate group significantly reduced the number of visible tumours, compared with the PBS group. High dose lysates enhanced the infiltration of inflammatory cells in the gut mucosa. <i>L. acidophilus</i> pre-treatment inhibited the production of IL-10 in macrophages in vitro. Treatment with lysates did not change TNF- $\alpha$ production. Lysates increased CD8+ and CD26L+ T cells in the mesenteric lymph.	This study was robust and scored well against the quality assessment criteria, but it did have its limitations. With the analysis and data collection methods, there were some drawbacks as there is no well-established method to obtain cytotoxic t cells led by lysates. Also, the link between inflammatory bowel disease (IBD) and colon cancer is studied in these experiments but, this is rarely seen (1% of cases). Although the study did acknowledge its flaws. <i>L. acidophilus</i> improved T cell immunity and overall showed to have positive immunomodulatory effects in this study. this study showed that lysates alone were enough to produce these positive effects.
Khan, et al.	32 Apc <sup>Min/+</sup> mice (6-8 weeks old).	The mice were divided into 4 groups, and for 8 weeks, received treatment. The groups received a control, <i>Ganoderma lucidum</i> polysaccharides (GLP) (750mg/kg), <i>Gynostemma pentaphyllum</i> (GpS) (300mg/kg) and combination of GLP and GpS (750mg/kg + 300mg/kg) single doses were given to the respective groups daily.	Enterobacterial repetitive intergenic consensus – polymerase chain reaction (ERIC-PCR) analysis on faecal samples. qRT-PCR to assess bacteria of the gut microbiome. Western blot analysis to identify N-cadherin, E-cadherin, iNOS, b-catenin, PSTAT3, p-Src, p-ERK (extracellular signal-regulated kinases), p-AKT/AKT (protein kinase B). Serum SCFA was measured using ultra-high performance	Colon tissue. Faecal sample	GLP and GpS reduced the number of polyps and polyp size. The amount of Paneth and goblet cells in the intestinal environment increased as well as their corresponding biomarkers p-lysozyme and <i>MUC2</i> gene. There was upregulated expression of E-cadherin and	This controlled clinical trial was very robust, especially with regard to its data collection methods, intervention integrity and analysis. Overall, this study scored highly against the quality assessment criteria. GLP and GpS have been shown in this study to modulate the gut microbiome and reduce the burden of polyps in Apc <sup>Min/+</sup> mice.

			liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF/MS). Macroscopic counting and measuring of polyps.		downregulation of N-cadherin in treated mice. Proinflammatory cytokines were significantly downregulated (IL-1 $\beta$ , INF- $\gamma$ , forkhead box P3 protein 'FOXP3' and TNF- $\alpha$ ) and anti-inflammatory cytokines were significantly up-regulated (IL-4, IL-10, IL-12 and IL-13). Lower levels of pERK and pARK were found in the GpS and GLP treated mice. GLP and GpS significantly reduced the abundance of harmful bacteria, mostly sulfide reducing bacteria. GLP and GpS also promoted SCFA producing bacteria.	Promising novel results.
Qamar, et al.	84 Male Wister rats (6-week-old).	Rats were divided into 7 groups, 12 per group. In group 1 (G1) the controls were fed a standard diet. G2 were the DMH group. G3-G7 were treated with the prebiotic galacto-oligosaccharides (GOS) (G3 received 76mg, G4 received 114mg and G5 received 151mg). G6 was given inulin and G7 received combination of GOS and inulin w/ basal diet. The prebiotics were given for 16 weeks. After 4 weeks of prebiotic dosages, G2-G7 received DMH	The pH of cecal and faecal digesta was measured using a microelectrode and pH/ION metre. Gas chromatography to measure SCFA in faeces. Methylene blue staining to count aberrant crypt foci under the light microscope.	Proximal, middle, and distal colon. Faecal samples.	Groups 3-5 given the GOS treatment showed resistance to DMH-induced body weight loss. Inulin, at a dose of 114mg exerted a better effect on body weight recovery than GOS treatment at the same dose. Faecal and cecal pH was not significantly different among all the groups. Group 2 showed higher levels	This study had very robust data collection methods and intervention design. Overall, this study scored well against the quality assessment criteria. The study highlights the potential of GOS as a prebiotic treatment for aberrant crypt foci, a unique angle in the literature for the prevention of CRC research.

			injections twice a week for 2 weeks to induce cancer.			of enzyme activities in the cecum and faecal content ( $\beta$ -glucuronidase, and $\beta$ -glucosidase). Groups 5-7 showed higher levels of SCFA production, especially acetate, propionate and butyrate, and reductions in ammonia.
Dos Santos Cruz, et al.	45, healthy male C57BL/6J mice (8 weeks old).	Mice divided into 3 groups to receive the control diet, probiotic (VSL#3 at $2.25 \times 10^9$ cfu/0.1mL) and synbiotic (VSL#3 $2.25 \times 10^9$ cfu/0.1mL + yacon supplement at 6% FOS and inulin) for 13 weeks. Pre-neoplastic lesions were induced at week 3 with DMH.	Evaluation of $\beta$ -glucuronidase activity, microbiota composition by qRT-PCR, cytokine profile by flow cytometry and SCFA levels with high-performance liquid chromatography (HPLC). Aberrant crypt foci were counted on the mice's colons.	Faecal samples. Colon.	The synbiotics group reduced aberrant crypt foci by 38.1% compared to the control. The synbiotics group also exuded a slightly lower pH. Both the probiotic and synbiotic groups had an increase in IL-4, but the synbiotic group had a significantly lower level of TNF. There were no significant differences in the levels of IL-6, IL-10, IL-17 and IFN- $\gamma$ . Animals receiving the synbiotic displayed reduction in $\beta$ -glucuronidase activity. The synbiotic group had higher concentrations of acetic, propionic, and butyric acids, compared to the control and probiotic group. All the groups showed significant differences in bacterial	The study design and data collection method were the strong points of this study. many different tests were used to measure the exposure. Overall, this study included very robust experiments and scored highly against the quality assessment criteria. The use of synbiotics in this study appeared to have positive effects against the progression of CRC. The yacon based supplement details were not available as it's undergoing a patent. We don't know if it's the combination of the VSL#3 probiotic or the yacon element alone causing these effects. Nonetheless, this study still yielded promising results.

Saito, et al.	Male 8 week old CPC;Apc mice (8 weeks old).	1% Dextran sodium sulfate (DSS) was given to the mice to induce acute colitis for 7 days. Synbiotics, probiotics ( <i>Lactobacillus casei</i> and <i>Bifidobacterium breve</i> ) and prebiotics were orally consumed from week 7 to week 20.	H&E to stain colon samples to examine inflammation, crypt damage and ulceration. qRT-PCR to identify inflammatory markers: IL-6, STAT3, NF-κB, PGE-2, COX-2 (cyclooxygenase-2) and TNF-α. qRT-PCR was also used to examine the bacteriological state of faeces.	Colon samples. Faecal samples.	community structure. There was a significant reduction in <i>Bacteroidetes</i> in the synbiotics group compared to the control and probiotics group. There was no significant reduction in tumour number in the probiotics and prebiotics group compared with the DSS control group, nor was there a difference in max tumour diameter. There was a significant reduction (45%) in tumour number in the synbiotics group. Treatment with synbiotics decreased inflammation compared to DSS alone. No significant differences in microbial bacteria. Synbiotic treatment significantly reversed the increase in pro-inflammatory markers observed in DSS treated mice (STAT3 by 41%, COX-2 by 66%, and TNF-α by 73%). <i>Lactobacillus casei</i> and <i>Bifidobacterium breve</i> were present in the microbiota of	This study scores well against the quality assessment criteria. This study did have some limitations, however. The combination of probiotics and prebiotic strains was only 1 of many possible combinations, and the study looked at already established effective probiotics and prebiotics. These were acknowledged, however. The data in this study showed the inhibitory effects of the synbiotics, suppressing the tumours in the DSS induced mouse model. The results of this study only show a significant impact on the mice already induced with DSS.
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Hetland, et al.	46 A/J Min/+ mice were used.	Mice were split into 2 groups, either receiving tap water with 10% Andosan ( <i>Agaricus blazei</i> , <i>Hericium erinaceus</i> and <i>Grifola frondose</i> ) mushroom extract or just tap water (control). This was given to the mice for 22 weeks.	Serum cytokines (IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, TNF- $\alpha$ , and IFN- $\gamma$ and Granulocyte macrophage colony-stimulating factor 'GM-CSF') measured using Luminex multiplex analysis. Flow cytometry for measuring the percentage of apoptotic cells.	Intestine samples.	the treatment groups but not in the control. The Andosan treated group exuded reduced cell viability in the Caco-2 cell lines, in vitro. The treated group has significantly reduced the size and number of tumours (60% reduction in tumour load) compared to the control. There was also a significant increase in anti-tumour Th1 type cytokines IL-12p70 and pro-inflammatory cytokines IL-1b, MCP-1 and TNF- $\alpha$ . However, Th2 and Th17 cytokines responses were not affected.	This controlled clinical trial scored well against the quality assessment criteria, with very detailed data collection methods. The data in this study shows promising results for the Andosan extract. That the extract protects against the development of colorectal cancer. In vitro, Andosan reduced cell viability, in vivo, the prebiotic exuded downregulation in some proinflammatory markers (although not all), reduced tumour load and have antiproliferative effects.
Tiptiri-Kourpeti, et al.	Administration of <i>Lactobacillus casei</i> and other probiotic bacteria on murine (CT26) and human (HT29) colon carcinoma cells. 40 female BALB/c mice (6-8 weeks old).	Incubation of CT26 and HT29 cell lines with live <i>L. casei</i> was carried out in the SRB assay (sulforhodamine B assay). Mice were separated into 2 groups (10 mice per group). One group received the control, and the other received oral daily administration of 1.0x10 <sup>9</sup> cfu/mL of <i>Lactobacillus casei</i> for 13 days. From day 10, 5.0x10 <sup>6</sup> CT26 cells per mouse were injected to induce tumour growth. TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) detection by immunofluorescence	Cell viability assays. HPLC analysis to measure the concentration of SCFAs. Annexin V and Propidium Iodide staining to measure apoptosis. Macroscopic measurement of tumour size.	Tumour. Tumour biopsy.	In vitro, <i>L. casei</i> , reduced the growth of CT26 cells by 52% at concentrations of 10 <sup>9</sup> cfu/mL, and by 78% for HT29 cells. <i>Lactobacillus casei</i> induced apoptosis in both cell lines from the annexin V and propidium iodide staining. In vivo, the <i>L. casei</i> significantly reduced tumour volume by 80%. <i>L. casei</i> adhered to colon cancer cells. In CT26 cells a higher gene expression of TRAIL was	This controlled clinical trial was scored well against the assessment criteria. Its study design and collection methods were very thorough. In this study, many anti-neoplastic effects of <i>L. casei</i> were observed. <i>L. casei</i> adhered to colon cancer cells, reduced colon cancer cell viability and induced apoptosis in vitro. <i>L. casei</i> also downregulated the expression of cyclin D1 and BIRC5a whilst upregulating the expression of TRAIL, which is significant because its overexpression may be linked to the induction of apoptosis, whilst

observed 60-fold, compared to non-treated cells. Downregulation of BIRC5a (aka surviving) and cyclin D1 was also observed. Increased expressions of TRAIL were also observed in western blot analysis. In the mice model, the treated mice grew smaller tumours in 90% of cases. Tumours from the treated mice showed higher expressions of TRAIL and lower survivin levels.

This table depicts ten different animal studies. The studies include models of induced cancers in the test subjects, and in every study, there is some positive benefit reported from the use of probiotics/prebiotics. Like the human studies, there were reported reductions in pro-inflammatory cytokines and increased anti-inflammatory cytokines in the models, reduced cell proliferation and induced apoptosis.

4.4 In Vitro Studies

Table 6. Intestinal microbiota modifications from probiotic/prebiotic in vitro studies

Study	Probiotic/ prebiotic	Study design & Intervention	Primary ends point/ Technique used	Results	Appraisal, Commentary/Criticisms and Remarks
Allsopp, et al.	<i>Agave angustifolia</i> Raw derived branched fructans	SHIME 5 vessel was utilised modelling proximal, transverse, and distal colon. The Model was inoculated with the faecal samples of male volunteers to create a microbiome environment. After a 2-week stabilisation period and 2-week pre-treatment control <i>Agave</i> fructans (AGV) were added for 3 weeks. The starch from the pre-treatment was replaced with AGV (2g/day). After 3 weeks of AGV, the starch nutritional media was re-introduced for a 2-week post-treatment	Plate counting, enumerating CFU, and analysing microbial community. Gas chromatography to identify SCFAs. MTT colorimetric assay to identify cell toxicity of the Caco-2 cells. TER (transepithelial resistance) assay was used to measure the electrical resistance of Caco-2 cells. Single gel	From the plate counting, it was determined that AGV treatment increased Bifidobacteria populations in all 3 vessels (especially in the ascending and transverse areas). The Bifidobacteria levels dropped again post-treatment. Similar trends were seen in Lactobacilli also. No significant differences were determined for Clostridia and <i>Enterococci</i> . Total SCFA increased during AGV supplementation in the proximal,	This paper scored highly across all the quality assessment criteria, it had a very robust study design, analyses, and data collection methods. The AGV prebiotic supplement showed promising results, increasing the amount of beneficial probiotic bacteria in the model. The results indicate that the amount of SCFA's also increased in the model. This paper further reinforces the use of fructo-oligosaccharides as prebiotics and their anti-cancer benefits.

		control.	electrophoresis to measure the extent of DNA damage in the Caco-2 cells.	transverse, and distal colon. AGV treatment did not significantly increase ammonia. The MTT assay did not show cytotoxic effects of SHIME supernatant samples from the vessels. TER assay revealed that TER value increased after 24hr post AGV treatment. No significant anti-genotoxic effects of any of the SHIME supernatants.	
Nuridin, et al.	Green Cincau extract from <i>Premna oblonifolia</i> Merr to be tested as a source of dietary fibre in Cell culture with Caco-2 cell lines.	In vitro anaerobic culture was carried out using CSIRO protocol (pectin, inulin, cellulose, pectin-cellulose, inulin-cellulose, pectin-inulin) and Cincau extract. Fermentation media was added to each dietary fibre source. Faecal inoculums were prepared and mixed with dietary fibre sources. The processes were carried out in an anaerobic chamber. The experiment was conducted on human Caco-2 cells. The experiment was to see what effect the dietary fibre sources would have on SCFA production.	Gas chromatography for SCFA analysis. MTT proliferation assay. Alkaline phosphate activity assay. Caspase-3/7 assay to measure enzyme activity. Lactate dehydrogenase (LDH) assay.	Cell cultures with green Cincau extract as the dietary fibre sources significantly increased levels of SCFA, specifically acetate and propionate compared to the faecal blank. Caco-2 cell viability was reduced after incubation with the dietary fibre supernatant after 20% of all the dietary fibre sources, including Cincau, except for cellulose. Inulin was the most effective at reduce cell viability. Alkaline phosphatase enzyme levels are used to measure cell differentiation. No dietary fibre source increased the enzyme levels, but some unexpectedly decreased the enzyme level (i.e., inulin, pectin-inulin), but Cincau had similar alkaline phosphatase levels to the faecal blank. Caspase-3 and caspase-7 are key effectors of apoptosis, their activity was measured in Caco-2 cells. Cincau suppressed caspase activity whereas pectin, inulin, pectin-cellulose, and pectin-inulin increased it.	This study was very thorough. It tested lots of different parameters to validate its findings. Overall, this paper scored highly against the quality assessment criteria. The other dietary fibre sources also increased SCFA production, some more or equal to Cincau extract (pectin-inulin, inulin, only cellulose seemed not to produce a significant increase in SCFAs). Cincau did not produce a significant increase in butyrate. Cincau did not appear to increase the activity of caspase-3 and caspase-7 enzymes or increase alkaline phosphatase levels, as the other dietary fibre sources did, but Cincau extract still appeared to have some helpful benefits. This paper demonstrated how this new prebiotic supplement may have some anti-neoplastic benefits, i.e., by increasing SCFA production, but the benefit is not as profound as other studied prebiotics (inulin etc).
Yun, et al.	Testing agarose-derived sugars' (AHG, NeoDP2, AgaDP3, NeoDP4, AgaDP5, and NeoDP6).	Different agar-derived sugars were made by different enzymatic reactions. The sugars	Cell growth was measured using optical density at 600nm. MTT	Only AgaDP3 increased the growth of <i>B. infantis</i> , but this growth was lower	This article scored fairly against the quality assessment criteria. Some of the results seemed to

	effect on the growth of <i>Bifidobacterium infantis</i> .	were cultured with <i>Bifidobacterium infantis</i> 15697. This was compared to the control of glucose, galactose and 2'-fucosyllactose.	assay to measure the viability of human colorectal carcinoma (HCT-116) cells. Western blot to measure in vitro anti-colon cancer activity of 3,6-anhydro-L-galactose (AHG).	compared to growth with glucose. Only AHG inhibited the growth of HCT-116 human colon cancer cells significantly and reduced their viability. AHG also reduced the level of anti-apoptotic proteins Bcl-2 and Bcl-xL and increased the expression of pro-apoptotic protein Bax. Tumour suppressor protein P53 was also induced by AHG.	indicate that the agar derived sugars aren't as effective, this could be due to flaws in the experimental approach. The experiment utilised different agar-derived sugars from red seaweed as in the gut, the red seaweed would be broken down into different sugars and therein exert their anti-neoplastic effects in the gut. This experiment showed that AHG significantly inhibited the proliferation of colon cancer cells, and induced apoptosis in cells. This study also showed that AgaDP3 was consumed by probiotic Bifidobacteria ( <i>B. infantis</i> and <i>B. kashiwanohense</i> ).
Grimoud, et al.	Lactic acid bacteria ( <i>Bifidobacterium bifidum</i> 02, 20 and <i>Bifidobacterium pseudocatenulatum</i> , <i>Lactobacillus farciminis</i> CIP 103136 <i>Lactococcus lactis</i> , <i>Pediococcus acidilactici</i> ), gluco-oligosaccharides, or symbiotic combination of the two.	HT-29 cells were seeded in 24 well plates, cultured for 7 days, and then inoculated with probiotic bacteria ( <i>Bifidobacterium bifidum</i> 02, 20 and <i>Bifidobacterium pseudocatenulatum</i> ), sterile filtered GOS (oligoaternal and aligodextran) or a combination of probiotics and GOS.	ELISA assays to test for secreted IL-8 quantities and NF-κB. Tests to quantify NF-κB in transgenic cell models – AGIR test. XTT assays to quantify cell proliferation of HT-29 cell lines. RT-PCR to measure cell differentiation of HT-cell lines.	Probiotics of GOS reduced secretion of IL-8 or NK-κB activation when cultured with inflammatory stimulated HT-29 cells. <i>B. pseudocatenulatum</i> , <i>Lactobacillus helveticus</i> and <i>L. lactis</i> caused a dramatic reduction in IL-8 and activation of NF-κB. <i>B. breve</i> , <i>L. farciminis</i> , and <i>L. rhamnosus</i> induced a significant decrease in the transgenic Caco-2 cell lines, compared to observations made in the HT-29 model. GOS did not induce variation in HT-29 cell proliferation. <i>B. breve</i> , <i>L. rhamnosus</i> and <i>L. lactis</i> decreased proliferation by more than 20%.	This study was robust, it tested many different parameters to reinforce its findings. Overall, this paper scored well against the quality assessment criteria. In the first cell culture on HT-29 cells, 5 of the strains were able to dramatically inhibit NF-κB activation and IL-8 secretion. The second model of transgenic Caco-2 cell line culture was used, which also resulted in strong anti NF-κB effects for some strains of probiotic bacteria. Overall, there are positive anti-inflammatory properties of some of the tested lactic acid probiotic bacteria that may be beneficial in colorectal cancer patients. The tests were all in vitro however.
Konishi, et al.	<i>Lactobacillus Casei</i> ATCC334 on colon cancer cell lines and ferrichrome produced by <i>L. casei</i> is the key effector molecule, (which mediates apoptosis by the JNK pathway).	Ferrichrome was identified as the tumour suppressing molecule in the tested probiotic bacteria by testing the extracted supernatants from <i>Lactobacillus GG</i> ATCC53103, <i>L. casei</i> ATCC334, <i>Lactobacillus coryniformis</i> ATCC25600 and	SRB assay with ferrichrome, and lipocalin-2 or ferrichrome permease (binding proteins) to test ferrichrome's anticancer ability was used.	SRB assays of ferrichrome at >100ng/ml reduced cellular proliferation of Caco2 and SW620 cells. The tumour suppressive effect was reduced in cases with the ferrichrome permease binding protein which supports that ferrichrome has a	This study was extremely exhaustive, carrying 15 different experimental tests on different parameters, to validate these papers' findings. This paper scored very highly against the quality assessment criteria. The effect of ferrichrome on non-cancerous cells was greater than that of existing anti-cancer

		<i>Lactobacillus fermentis</i> .		tumour suppressive effect. No significant effects on non-cancerous cells were observed suggesting that ferrichrome is safe to use on non-cancerous cells.	treatments such as 5-fluorouracil and cisplatin. The tests revealed that ferrichrome was effective at reducing tumour growth whilst having minimal effect on non-cancerous cells. This is the only paper included in this review where the isolated molecule from probiotic bacteria has been identified.
Ichim, Kesari, & Shafer.	A blend of <i>Lactobacillus</i> and <i>Bifidobacterium</i> genera was used.	SHIME model was used, to replicate the human GI. 5-FU/vancomycin was added to emulate cancer treatment, with probiotics to identify the potential benefits this may have if current cancer treatment was supplemented with probiotic bacteria. The short-chain fatty acid production was monitored in the proximal, and distal colon reactors. Probiotic bacteria were added to the SHIME vessels to emulate curative and preventative administration.	qPCR to identify changes in microbial composition.	Treatment with the probiotic mixture did increase SCFA levels in the preventative experiment, although it did not recover the SCFA during the 5-FU/vancomycin period. Certain strains of <i>Lactobacillus casei</i> and <i>Bifidobacterium</i> had beneficial effects, <i>Lactobacillus</i> levels recovered after the administration of the probiotic with digestive enzyme supplement. Treatment resulted in faster recovery. There was also faster recovery of Bacteroidetes after 5-FU treatment. Curative treatment with the supplement increased the microbial recovery and diversity in both proximal and distal colons. The treatment also promoted metabolites that decreased pro-inflammatory IL-6 concentrations.	This paper was thorough and overall scored well against the quality assessment criteria. This experiment used a unique model to assess the benefit of probiotics with pre-existing cancer treatment 5-FU/vancomycin in a SHIME-model. The study did show that the probiotics have a beneficial effect, reversing the 5-FU/vancomycin induced dysbiosis in all tested parameters except SCFA levels. Overall, very promising results and could encourage the potential use of probiotic bacteria alongside cancer treatment in the future.

This table depicts six studies and outlines the probiotic/prebiotic in use, the intervention, the methods of measuring the primary endpoints and the results. These studies reported positive effects of the probiotic/prebiotic agents In vitro. Many of the parameters measured were also seen in animal and human studies.

## 5. Discussion

### 5.1 GI and Microbiome

The GI has around 200-400m<sup>2</sup> surface area exposed to environmental stressors, microflora, and pathogens, with the healthy human microbiome consisting of *Firmicutes*, *Bacteroidetes*, then in lesser amounts, *Actinobacteria* and *Verrucomicrobia* (Jandhyala et al., 2015). It was noted that treatment with the wheat bran extract prebiotic containing arabinoxylan-oligosaccharides, significantly increased the microbial diversity in the study with 20 healthy volunteers (Windey et al., 2015). This is significant for WBE and other similar prebiotics, these could potentially act as preventative measures against dysbiosis during CRC treatment. In another study of 22 patients, the probiotic bacteria *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis* significantly increased the richness and diversity of the patient's microbiome (Gao et al., 2015). This microbial

recovery was observed in CRC patients about to undergo a colectomy, meaning the probiotics may be effective, taken as a pre-operative measure for example. Similar trends were also seen in vitro, (Allsopp et al., 2013), with *Agave angustifolia* increasing Bifidobacteria populations in the SHIME model after 3 weeks. In another experiment also utilising the SHIME model, a blend of *Lactobacillus* and *Bifidobacterium* genera increased microbial recovery and diversity in the model treated with vancomycin (Ichim, Kesari and Shafer, 2018). This is similar to the animal studies, where purified saponin compounds from *Gynostemma pentaphyllum* were observed to increase the microbiome bacterial diversity of *Apc*<sup>Min/+</sup> mice (Huang et al., 2017). The SHIME studies are particularly interesting, as they demonstrate the ability of prebiotics and probiotic strains to positively restore the simulated microbiome environment. And despite the drawbacks, we still see parallels with the animal and human studies, where the probiotics/prebiotics were able to restore microbial richness and diversity. From these studies, we can see the anti-cancerous benefits observed in vitro also has benefits in humans, which is promising, and we could see these probiotics/prebiotics used with CRC therapy in the future.

The perimeter of the intestinal lumen is a thick mucus layer containing mucosal IgA antibodies which helps form a barrier between the epithelial cells lining and the microbial cells, and in a healthy state, the immune response to intestinal microbiota is compartmentalised to the mucosal surface (Zheng, Liwinski and Elinav, 2020). The MUC2 mucin protein is responsible for organising the mucus barrier and creating protective shielding, but also it encourages enteric dendritic cells to an anti-inflammatory state. Interestingly, it was observed in the 8-week study with *Apc*<sup>Min/+</sup> mice, treated with *Ganoderma lucidum* polysaccharides, and *Gynostemma pentaphyllum*, that there was an increase in the MUC2 gene (Khan et al., 2019). So as explained previously, by encouraging the dendritic cells to an anti-inflammatory state, *Ganoderma lucidum* and *Gynostemma pentaphyllum* may be responsible for reducing polyp size and number, and increasing Paneth and Goblet cells in mice's colon tissue.

The epithelium lining of the intestinal lumen uses toll-like receptors and NOD-like receptors that detect PAMPs (pathogen-associated molecular patterns) which are used to respond to bacterial pathogens or harmless commensal microflora with an immune response. This is either an inflammatory response or immune tolerance signalling (Sansonetti and Medzhitov, 2009). The gut microbiota can influence the immune response, nutrient acquisition and gut permeability, which is due to the ability of the microbiota to change gene expression in the host (Richards et al., 2019). Typically with gut dysbiosis, we see a reduced diversity of pathogenic and commensal bacteria, meaning that we see the loss of beneficial bacteria, the overgrowth of potentially harmful bacteria and the loss of overall bacterial diversity (DeGruttola et al., 2016). Often this is accompanied by changes in bacterial metabolic activities. Along with CRC, dysbiosis has been associated with other diseases i.e., inflammatory bowel disease, type 1 and type 2 diabetes and obesity. In the case of CRC, this dysbiosis often results in a reduction in the amount of butyrate-producing bacterial species (i.e., Proteobacteria, Bifidobacteria etc). The gut microbiome is important in upregulating proteins and helps maintain tight junctions along the intestinal epithelium. So often in dysbiosis, we see the formation of gaps in the epithelium, making it more permeable. This increases the passage of microbiota and metabolic products into the lamina propria which can initiate an immune response, increasing pro-inflammatory cytokines.

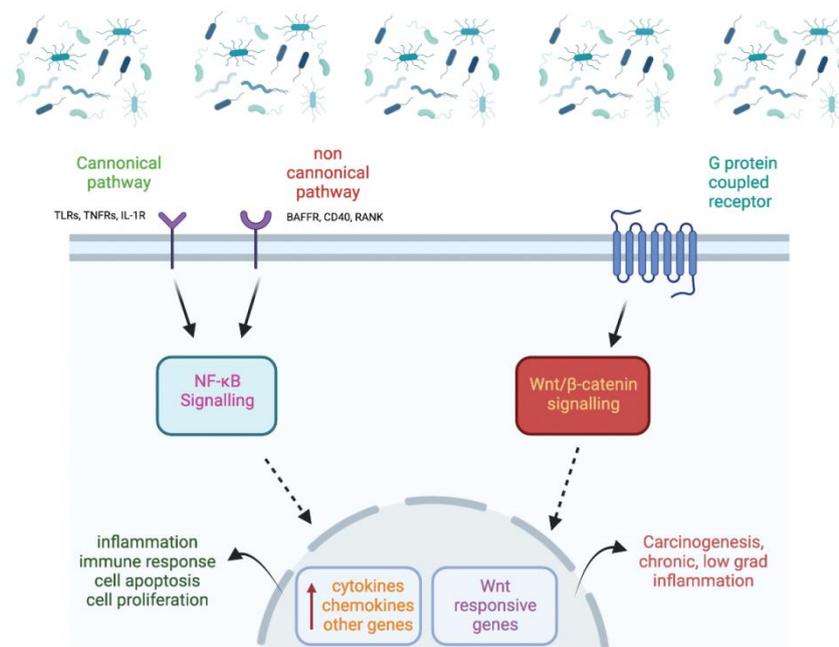


Figure 2. Figure 2 depicts two separate pathways that have been hypothesised to take place during gut dysbiosis. The NF- $\kappa$ B pathways and the wnt/ $\beta$ -catenin pathway link gut dysbiosis to CRC

Figure 2 depicts how dysbiosis may elicit downstream signalling to induce inflammation, cell proliferation, and carcinogenesis, which is the case in CRC. Species like *Fusobacterium*, *Leptotrichia* and *Campylobacter* are higher in individuals with tumour overexpression. *Fusobacterium nucleatum* is much higher in individuals with CRC (Brennan and Garrett, 2019), and it may be instances like this whereupon infection with pathogenic bacteria, the NF- $\kappa$ B pathway is activated. Normally, in healthy tissue, the NF- $\kappa$ B pathway is involved in innate immune response and intestinal homeostasis and is responsible for generating new tissue to heal wounds. Thus, overactive expression of NF- $\kappa$ B signalling may lead to chronic inflammation, and eventually, increase the likelihood of polyp formation. It was found that *Bifidobacterium pseudocatenulatum*, *Lactobacillus helveticus*, and *Lactobacillus lactis*, dramatically reduced the NF- $\kappa$ B activation in HT-29 cancer cell lines (Saito et al., 2019). So, these probiotics strains, appear to reduce a key inflammatory pathway observed in CRC, the same pathways exist in vivo, and so if these same pathways are downregulated in mice and human subjects, this could be very promising.

Toll-like receptors and NOD-like receptors bind with virulence factors on pathogenic bacteria, such as CagA and peptidoglycan (Peng et al., 2020). The pathway is activated by phosphorylating I $\kappa$ B via, NF- $\kappa$ B inhibitor kinase (IKK). This induces downstream signalling, which promotes inflammation, cell proliferation and tumorigenesis. A similar sequence of events follows for the Wnt/ $\beta$ -catenin pathway, but in CRC, gut dysbiosis causes constitutive activation of the pathway, resulting in the overexpression of oncogenes. And we expect this to happen in chronically inflamed tissues (this increases the likelihood of tumour formation).

The probiotics and prebiotics have been shown to have immunomodulatory effects against CRC, which has been hypothesised to occur via downregulating the Wnt/ $\beta$ -catenin and NF- $\kappa$ B pathways. This is valuable to us, as we can somewhat explain the mechanism by which probiotics/prebiotics exhibit their immunomodulatory effects, although the details of how this mechanism works are still unclear, it still adds value.

## 5.2 SCFAs

The way probiotics and prebiotics affect the production of short-chain fatty acids is important to this discussion, as SCFA's have shown immunomodulatory effects on the host's microbiome that help maintain intestinal homeostasis. These functions are coordinated by mechanisms including histone deacetylase inhibition, G-protein coupled receptor signalling, and acetyl-CoA production (Kim, 2021). It is through these mechanisms that SCFAs can promote anti-inflammatory immune response and immune tolerance. Allsopp, et al (2013) researched the impact of isolated branched fructans from *Agave angustifolia*, in the SHIME in vitro model, whereby there was a marked increase in SCFAs; acetic acid, propionate and butyric acid (by varying amounts). Similar increases in

SCFAs have been observed in the 2019 study with Khan, et al, the 2016 study with Qamar, et al, the 2020 study with Dos Santos Cruz, et al, the 2017 study with Nurdin, et al, and the SHIME- 5-FU/vancomycin study conducted in 2018 by Ichim, Kesari & Shafer. Allsopp's paper investigated the benefits of branched fructans, which are drastically different from the well-studied inulin-type fructans and their linear structure. The AGV fermentation was observed to have a more sustained fermentation pattern, instead of the harmful proteolytic fermentation patterns observed elsewhere. This is compelling, as it could indicate a long-term anti-inflammatory response, which would be beneficial to many CRC patients experiencing GI inflammation. Long term sufferers of inflammatory bowel disease are at higher risk of developing CRC – they may benefit from SCFA producing prebiotics/probiotics. In Ichim, Kesari, & Shafer's study, a blend of *Lactobacillus* and *Bifidobacterium* species was used in the SHIME model simulating cancer treatment with 5-Fluorouracil/vancomycin. The probiotic blend successfully improved recovery time, and it restored microbial diversity in the gut faster than the control and promoted the production of metabolites that have been shown to decrease pro-inflammatory cytokine concentrations such as IL-6 in other studies. This positive immunomodulatory effect is likely due to the increase in SCFA levels, and thus further illustrates the benefit of *Lactobacillus* and *Bifidobacterium* in the SHIME model, we now only need to see these benefits in human studies.

In the aforementioned studies, positive anti-neoplastic effects due to the increase in SCFA's have been attributed to these specific probiotic/prebiotics: *Gynostemma pentaphyllum* (GpS), *Ganoderma lucidum* (GLP), galactooligosaccharides that contain  $\beta$ -1,6 and  $\beta$ -1,3 linkages, the VSL#3 polybiotic (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus delbrueckii* (subspecies) *bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium infantis*), and green cincau extract from *Premna oblongifolia* Merr. The studies have demonstrated the ability of these probiotics/prebiotics to increase SCFA's and create an anti-inflammatory response in some in vitro models, and thus have promising potential for further testing on human subjects in the future.

### 5.3 Immunomodulation

As mentioned before, SCFA can modulate an immune response, which is often seen as a reduction in pro-inflammatory cytokines. The study testing the effect of a six probiotic strain capsule on 52 colorectal cancer patients by Zaharuddin, et al (2019), showed a significant reduction in the levels of TNF- $\alpha$ , IL-10, IL-12, IL-17A, IL-17C, IL-22, and IL-6, which are inflammatory cytokines. The polybiotic capsule contained strains of *Lactobacillus acidophilus*, *Lactobacillus lactis*, *Lactobacillus casei*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Bifidobacterium infantis*. The Kotzampassi, et al (2015) study demonstrated that a different polybiotic containing *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Bifidobacterium lactis* and *Saccharomyces boulardii*, was able to modulate the expression of TNF, IL-6 and the *SOCS3* gene. There was a strong correlation between the bacteria and the inflammatory markers. The modulation of *SOCS3* is particularly interesting, as *SOCS3* is known to control overwhelming cytokine responses. This particular pathway could explain the anti-inflammatory effects observed in other studies. The 2017 study by Huang, et al, provided evidence to suggest that a saponin compound gypenoside (Rd) from *Gynostemma pentaphyllum* significantly decreased the amount of pro-inflammatory cytokines IL-1 $\beta$ , IL-6, IL-12, IL-177 and IL-23. It also decreased iNOS which has been found in high levels in human CRC cells under the inflammatory state (Wang et al., 2020). CXCL10 (interferon- $\gamma$ -inducible protein 10) also decreased; it is an inflammatory chemokine that mediates immune response through leukocyte recruitment. This is significant as these same pathways and inflammatory cytokines exist in vivo, and it would be interesting to see if we can extrapolate the results and see potential benefits for human CRC patients. Anti-inflammatory effects have also been reported in the 2019 study by Zhou, et al whereby DSS (carcinogen) induced mice were treated with *L. acidophilus* lysates. The group that received a high dose of the probiotic ( $2 \times 10^7$  cfu) every other day for 5 weeks, experienced a decrease in IL-6 production and a reduced number of visible tumours. The lysates also increased CD8+ and CD26L+ T cells in the lymph. As was mentioned before, the 2019 study by Khan, et al promoted SCFA producing bacteria, but the study also showed that IL-1 $\beta$ , IFN- $\gamma$ , FOXP3 and TNF- $\alpha$  cytokines were downregulated by *Ganoderma lucidum* (GLP) and *Gynostemma pentaphyllum* (GpS) polysaccharides in Apc<sup>Min/+</sup> mice. In the study conducted by Saito, et al (2019), the use of a symbiotic blend of *Lactobacillus casei* and *Bifidobacterium breve* reversed increases in pro-inflammatory markers like STAT3 (by 41%), COX-2 (by 66%) and TNF- $\alpha$  (by 73%). These findings mean we can explain how the prebiotics/probiotics work on a cellular level and build upon the other observed effects, i.e., changes in the SCFA levels and microbial diversity. Other positive effects that probiotics and prebiotics have on inflammatory responses are reported in Hetland, et al (2016), where Andosan (from *Agaricus blazei*, *Hericeum erinaceus* and *Grifola*) increased anti-tumour Th1 type cytokines IL-12p70. This, like the other studies reporting immunomodulatory effects, reiterates the potential of these prebiotics. Reduced secretion of IL-8 was

observed in a study by Grimoud, et al (2010), caused by *Bifidobacterium pseudocatenulatum*, *Lactobacillus helveticus* and *L. lactis* in HT-29 cells. This experiment also observed a 20% reduction in cell proliferation. All these results are impressive and have the potential to develop novel treatments for sufferers of IBD, CRC and other inflammatory diseases affecting joints, and kidneys etc. Despite the studies' limitations, these results may be the start of a promising breakthrough. These studies' tested an array of different parameters, but the recurring theme was that probiotics and prebiotics significantly lowered the levels of pro-inflammatory markers, lowered their expression, and increased anti-inflammatory markers.

#### 5.4 Decreased Proliferation and Tumour Load

Many of the papers included in this review report a decrease in tumour load (i.e., tumour size and the total number of tumours) resulting from a decrease in proliferation and increase in apoptosis. one hypothesised pathway that describes apoptosis is the JNK pathway. Konishi, et al (2016) proposed this after isolating the active component from supernatants of *Lactobacillus GG*, *Lactobacillus casei*, *Lactobacillus coryniformis* and *Lactobacillus fermentis*, which was found to be ferrichrome. This isolated ferrichrome supposedly activates the ER stress response JNK pathway and thus results in apoptosis (Dhanasekaran and Reddy, 2008). Via this pathway, ferrichrome was found to reduce the cellular proliferation of Caco-2 and SW620 cancer cells, whilst having no adverse effects on non-cancerous cells. This shows the ability of *Lactobacillus* to specifically target proliferating cancerous cell lines. In another study, *L. brevis* resulted in JNK activation (Angulo et al., 2011), by phosphorylating JNK which was observed by western blotting analysis.

A reduction in overall tumour load was reported in the in vitro study (of *Lactobacillus casei* and other probiotics) in murine (CT26) and human (HT29) colon carcinoma cells (Tiptiri-Kourpeti et al., 2016), which is significant as tumour load can be viewed as the 'real' anti-neoplastic observation as the other observed mechanisms work to produce the effect of reducing tumour load.

In another study, tumour growth was inhibited by *L. rhamnosus* in female BALB/c rats when administered the probiotic at  $1.0 \times 10^8$  cfu daily for 14 days, and then once a week for 3 weeks at  $1.0 \times 10^9$  cfu (Hu et al., 2015). White corn tortilla (WCT) and blue corn tortilla (BCT) had the effect of reducing the development of tumours by 77.5% more than the control in male Sprague-Dawley rats (Reynoso-Camacho et al., 2015). Also, the tumours in the tortilla treated groups in this study have significantly lower levels of K-ras and  $\beta$ -catenin (especially in the WCT and BCT groups). Similar decreases in tumour load or polyp formation were observed in the studies by Zhou, et al (2019), Saito, et al (2019), Hetland, et al (2016), Khan, et al (2019), and Frederich, et al (2011). So clearly, the different probiotics and prebiotics such as white corn tortilla, blue corn tortilla and a variety of *Lactobacillus* species possess some benefit, stopping the development of, or reducing, tumour load in these studies. It's interesting as the *Lactobacillus* species specifically, also correlated with the increase in microbiome diversity, increase SCFA production, and anti-inflammatory immunomodulatory effects as seen above. But these results in decreasing cell proliferation and tumour load are perhaps the most significant as tumour load is the cumulative result of the other observed effects.

#### 5.5 Enzyme Activity

$\beta$ -glucuronidase has been identified as a potential tumour biomarker, as it is believed to be present due to tumour expression. It is released from necrotic tumour tissues, and/or tumour infiltrating cells (Su et al., 2014). The level of  $\beta$ -glucuronidase was reduced after treatment with synbiotic VSL#3 and a yacon based supplement in an experiment with C57BL/6J mice (dos Santos Cruz et al., 2020). This along with a reduced prevalence of aberrant crypt foci (by 38.1%) and an increase in SCFAs indicated that the synbiotic treatment of VSL#3 and the yacon supplement was effective at regressing the effects of induced colorectal carcinoma in mice. Similarly, in the study conducted on Sprague-Dawley rats by Reynoso-Chamaco, et al (2015), the levels of the  $\beta$ -glucuronidase in the groups treated with BCT, WCT and YCT were much lower in the ceca compared to the control group. This means that BCT, WCT and YCT reduced a tumour biomarker, indicating the downregulation of the tumour pathway.

Similarly, in a few studies, the level of Glutathione-S-transferase (GST) was identified as the primary endpoint for investigating the positive effects of probiotics. GST's role in catalysing the conjugation of reduced glutathione to xenobiotic substrates is essential for protection against DNA damage and oxidative stress (Chatterjee and Gupta, 2018). An accepted mechanism in cancer prevention is the upregulation of detoxifying agents like GST. The 2015 study by Reynoso-Chamaco, et al, measured GST levels by using the bicinchoninic acid protein assay, showing that white corn tortilla increased the level of GST in the liver, and the yellow corn and blue corn tortilla groups raised GST in the colon, compared to the controls. GST was also studied in patients with familial adenomatous polyposis (Friederich et al., 2011), whereby the treatment appeared to effectively

increase GST activity. This was the case only when patients were treated with either VSL#3 or inulin, but not in combination. So here the 3 corn tortilla varieties and the VSL#3/inulin combination exert anti-neoplastic effects by upregulating GST levels.

Lactate dehydrogenase, LDH is a non-specific marker for cancer, but it's also increased in response to tissue injury and other disease states (Liu et al., 2016). The extracellular release of LDH was measured as an additional marker of necrotic cell death in Caco-2 cells in an experiment testing the anti-cancerous effects of cincau extract from *Premna oblongifolia* Merr. Less LDH was released from the cells treated with cincau, suggesting that the extract could protect cells against necrotic cell death, however, this alone could not tell us if cincau had anti-neoplastic effects.

Caspase-3 is known to coordinate apoptosis, as is caspase-7. Although the exact role of these enzymes in tumour progression is unclear, we know that they are key effectors of apoptosis. We can measure these enzymes' activity in colorectal cancer cell lines to determine the apoptotic activity in the cells. From the same study that tested the effects of cincau, the levels of caspase-3 and caspase-7 were examined using the Caspase-GloR 3/7 assay kit. Cincau did not have a significant effect on the caspase-3/caspase-7 activity (in fact the activity level was lower than the faecal blank) however, other dietary fibres (Pectin, Inulin, and a combination of the two) significantly increased the caspase-3/caspase-7 activity, indicating that the level of apoptotic cell death was higher, and so these other dietary fibre sources were effective at inducing death of the cancerous Caco-2 cells. Liu, et al (2016) utilised western blotting to identify the expression of cleaved caspase-3 to further clarify the effects of ferrichrome on apoptosis of the cancerous cells, as a secondary outcome measure. As cleaved-caspase-3 levels are a result of the JNK signalling pathway that induces apoptosis, higher levels of cleaved caspase-3 indicate ferrichrome's effectivity in inducing apoptosis in the cancerous cells. The level of cleaved caspase-3 was higher in the ferrichrome treated cells, reinforcing the effectivity of ferrichrome to induce apoptosis.

### 5.6 Cytotoxicity and Genotoxicity

Three studies included in this review contained experiments on the effect of the probiotic/prebiotic on cytotoxicity and/or genotoxicity. Faecal water genotoxicity is often assessed as an early marker for risk of CRC and is determined by the faecal water's ability to create breakages in the DNA (Windey et al., 2014). Faecal water cytotoxicity, on the other hand, reflects the faecal water's ability to induce cell death and often is seen at higher levels in CRC patients (as cell growth and thus, cell death levels are increased).

The 2015 study by Windey, et al, looked at the effect of wheat bran extract on faecal water cytotoxicity and genotoxicity, but to no avail. In this study, the effect of wheat bran extract on the cytotoxicity was measured using the WST-1 assay, and faecal water genotoxicity was measured with the comet assay, but the results were no different from the placebo. It has been shown that some probiotics can reduce the faecal water cytotoxicity and genotoxicity levels, but the WBE did not, this compound also did not produce results that indicate any significant anti-neoplastic activity. The investigation by Frederich, et al., similarly looked at the cytotoxicity of faecal water, which in their case reduced with the VSL#3/inulin supplementation in patients with familial adenomatous polyps and ileal pouch-anal anastomosis but increased with sulindac therapy and the combination of sulindac/VSL#3 + inulin. But results on genotoxicity and cytotoxicity may not be reliable parameters as they are often associated with only early CRC onset, and in the same study, VSL#3 correlated with lowered inflammatory markers (i.e., TNF) and increased GST activity among other effects. So, this data on genotoxicity/cytotoxicity may not be conducive enough to rule out probiotics as having no benefit. The WBE only showed modest benefit with regards to the other parameters, perhaps this particular prebiotic was not as effective as the others.

### 5.7 E-cadherin/N-cadherin Ratio and Gut Epithelial Barrier

E-cadherins are expressed on most epithelial tissue. It controls many different functions from maintaining tissue structure and integrity to maintaining homeostasis of epithelial tissue. The loss of E-cadherin is associated with an invasive undifferentiated phenotype common in many epithelial cancers (Araki et al., 2011). N-cadherin, which is expressed in mesenchymal cells, is involved in cell-to-cell adhesion. However, some cancer cells express N-cadherin promoting cellular invasion and motility. This shift in the E-cadherin/N-cadherin ratio is often characterised in cancer progression and is referred to as 'the cadherin switch.'

Probiotics and prebiotics are often tested for the upregulation of E-cadherin and downregulation of N-cadherin, to reverse the 'cadherin switch.'. Huang, et al (2017) tested the effect of prebiotic ginsenosides Rb3 and Rd: these were able to successfully reinstate the E-cadherin/N-cadherin ratio in *Apc*<sup>Min/+</sup> mice. Khan, et al (2019), carried out similar tests with *Gynostemma pentaphyllum* and *Ganoderma lucidum*, in which downregulation of E-cadherin and the upregulation of N-cadherin were observed, using western blot analysis in *Apc*<sup>Min/+</sup> mice.

These prebiotics in these studies successfully demonstrated their ability to reinstate the cadherin switch.

These two studies also looked at how the intestinal Paneth and goblet cells in the colon changed with colon cancer and with probiotic/prebiotic intervention. Paneth cells are responsible for a variety of functions including, secreting antimicrobial peptides such as  $\alpha$ -defensins and assisting undifferentiated stem columnar cells thus defending epithelial cell renewal (Gassler, 2017). The cells manage to mediate host-microbe interactions (i.e., they help regulate the intestinal microbe composition. E-cadherins also play a crucial role in Paneth cell maturation, so the upregulation of E-cadherin may correlate with an increase in Paneth cells. Goblet cells are involved primarily in secreting mucus. In both experiments, the treatment with prebiotics increased the presence of Paneth cells and Goblets cells which improved the epithelial barrier. The *Apc<sup>Min/+</sup>* mice used in both studies are typically characterised by a dysfunctional epithelial barrier, so the results from the study provide evidence that the prebiotics were able to help restore epithelial barrier function to some extent, which may mean less inflammation and better immunomodulatory regulation (Khan et al., 2019).

#### 5.8 Increase in Microbial Diversity and Decrease in ETBF Bacteria

Odamaki, et al (2012) conducted a study examining the effect of yoghurt supplemented with *Bifidobacterium longum*, *Lactobacillus delbrueckii bulgaricus*, *S. thermophilus*, and *L. lactis* on the microbiome in a group of 32 healthy volunteers. Specifically, this study looked at the ability of *B. longum* to lower enterotoxigenic *Bacteroides fragilis* (ETBF), which is associated with an increased risk of developing CRC. This study was not able to show clearly that *Bifidobacterium longum* BB536 had any effect on ETBF. Many other papers included in this review did look more generally at how the diversity of the microbiome was affected by prebiotics and probiotics, and in most cases, the probiotics/prebiotics increased lactic acid-producing bacteria and reduced bacteria associated with CRC development (i.e., *Helicobacter pylori*, *Fusobacterium nucleatum*, *Bacteroides fragilis*). Although the evidence on modulating ETBF levels was not conclusive, still these probiotics have prospects, indicating anti-neoplastic benefits via other mechanisms. Gao, et al (2015) tested the effects of a mixture of *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis* on the microbial flora of 22 colorectal cancer patients, and 11 healthy volunteers. After bioinformatics analysis of pyrosequencing data, Shannon and Simpson's indices were calculated as diversity estimators and Chao and ACE were calculated to estimate species richness. The results showed that probiotics successfully increased the richness and diversity of the mucosal microbes. There was also a significant reduction in *Peptostreptococcus*, *Comamonas*, and *Fusobacterium*, which typically have a higher prevalence in CRC patients (Long et al., 2019), although the exact mechanism explaining why is still unclear. Similar results are observed in the study by Huang, et al (2017) and the study by Ichim, Kesari, & Shafer (2018). This is consistent with some of the other findings from papers where prebiotics such as WBE and lactic acid producing bacteria were able to restore the microbial diversity in the gut in patients about to undergo colectomy surgery. Thus, these probiotics/prebiotics show their ability to increase the beneficial bacteria.

#### 5.9 Expression of T Cells and NK Cells

Giannoti, et al (2010) and Hu, et al (2015) used flow cytometry to measure the T cell infiltration. In the 2010 study, patients undergoing colorectal resection were given a dose of *Bifidobacterium longum* (BB536) or *Lactobacillus johnsonii* (La1). Both probiotics were able to increase the stimulation of T cells, specifically CD83, CD83-123, CD83-11c and CD-83-HLA-DR, which is significant because cytotoxic cells that express CD8 are very powerful effectors in anticancer immune response (Raskov et al., 2021), if not the most powerful. There was an increased proliferation in all lymphocytes except B cells in the groups receiving probiotics. In the 2015 study by Hu et al, conducted on female BALB/c mice, *L. plantarum* and *L. rhamnosus* were administered to test their anti-neoplastic effects against CT26 cells. *L. plantarum* managed to successfully inhibit tumour growth. This group also saw the increased infiltration of CD4+, CD8+ T cells and Natural killer CD3-CD49b+ cells.

#### 5.10 Broader Significance of Findings

The evidence in this review suggests that probiotics/prebiotics have potential therapeutic benefits. With the probiotics/prebiotics included, adding to a growing list of beneficial compounds and bacteria already established in the literature. We see that the probiotics and prebiotics can increase SCFA production, restore microbial composition, modulate anti-inflammatory response, increase T cell expression, cause apoptosis of cancerous cell lines, decrease tumour load, increase enzyme activity of GST and LDH, and reinstate the cadherin ratio. Although each of these observed effects by themselves does not automatically mean that the probiotic in question is anti-cancerous, it shows that these bacteria and derived compounds can affect processes associated with cancer development positively, in an isolated environment. Many of the probiotics, specifically the *Lactobacillus* species have shown to consistently affect these processes associated with CRC development

positively, by slowing down or reversing these processes. The cumulative benefit observed by these probiotics/prebiotics is promising and provides grounds for more research to replicate these observations in human trials, with a larger more diverse cohort. But for now, we know that probiotics/prebiotics have potential.

Future testing that builds off studies like these will answer more specific questions as we learn more about the microbiome i.e., how branched fructans have a more sustained level of fermentation, (which increases the level of SCFAs produced per unit time). All these findings will help expand the growing list of potentially beneficial probiotics and prebiotics, to aid the scientific development of this field of research and help us find a probiotic that is effective enough to use for the treatment of CRC and other cancers. Some probiotics included in these studies have already proven safe to use in cancer patients. But, with most of the experimental evidence coming from in vitro and animal studies, there is a demand for more studies including human colorectal cancer patients, especially now that the body of literature has ample evidence suggesting that these probiotics/prebiotics are beneficial for sufferers of CRC and other GI inflammatory diseases. We can see promising results from the studies (as outlined in 5.2-5.9) which support the hypothesis that prebiotics/probiotics may be beneficial, but the extent of their benefit is still not entirely clear. The extent of their benefit in CRC patients will only be realised with more research on human participants.

There are however limitations to this review. This review looks at a broad range of papers, conducted on humans, mice, and in vitro laboratory tests, whereas other studies may include just one type of participant/subject matter (i.e., only CRC patients, or over 50+ volunteers etc). Other reviews may look at one type of intervention, i.e., only cytotoxicity levels, only the change in SCFA levels etc. Reviews like these would be more focused and critical, there is also the opportunity to include and compare more studies as there is only one point of comparison. This review was kept relatively broad, enabling cross-comparison of different tests within the studies, fitting the narrative of this review better. Also, the literature on probiotics and colorectal cancer is still relatively new, so this review included studies thought to be a true reflection of the full body of literature. By doing this we can follow the narrative of probiotics/prebiotics from the in vitro tests and follow their testing in animal studies and finally in humans to assess if they still exude their beneficial properties at the human stage.

## 6. Conclusion and Recommendations

Evidence in this review, suggests that *L. acidophilus*, *L. Casei*, *L. lactis*, *B. breve* and *B. longum* have all-around positive anti-neoplastic effects such as positive immunomodulatory effects, increasing SCFAs, decreasing cell proliferation, effects on enzyme activity, and positive effects against faecal water genotoxicity and cytotoxicity, and increasing microbial diversity. The evidence also suggests that prebiotic *Gynostemma pentaphyllum* has especially positive immunomodulatory effects, and also has positive effects reversing the E-cadherin/N-cadherin switch. The evidence also suggests that the prebiotics and probiotics detailed in the results and discussion positively impact colorectal cancer patients undergoing colorectal surgery. It is clear from these findings that prebiotics/probiotics appear to have some benefit in certain circumstances – with limitations. The evidence reflects the benefit of probiotics/prebiotics in animal and, in vitro models extensively, and these findings were present, but not as obvious from the human studies. The evidence reported in the animal studies describing the positive anti-neoplastic effects of probiotics and prebiotics is similar to the human studies in establishing, positive changes in cytotoxicity, genotoxicity, activating T cells and natural killer cells which impart positive immunomodulatory effects on the patients. There is still a gap in the literature where more human trials are needed. Although there have been positive immune responses to the probiotics/prebiotics in humans, we still don't know how these will affect overall tumour load, which is of importance because ultimately these experiments are to find additional treatment for CRC, so finding probiotics and prebiotics that inhibit/regress tumour load as we have seen in mice would be the next step. Right now, a wider expanse of beneficial probiotics and prebiotics builds itself as more papers in the literature are published, which is favourable as it builds the foundation for future research, but research is needed to identify to what extent these bacteria and other compounds can benefit humans. Reviews like these could set the foundation for a future wherein we know which products and compounds are natural prebiotics, that we can source locally, so we can eat healthier and prevent chronic diseases like colorectal cancer. Additionally, we can use probiotics and prebiotics to help the treatment of existing colorectal cancer patients and ultimately, make the world a healthier place.

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