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## Galacto-oligosaccharides and Colorectal Cancer: Feeding our Intestinal Probiome

Jose M. Bruno-Barcena<sup>1</sup> and M. Andrea Azcarate-Peril<sup>2,\*</sup>

<sup>1</sup>Department of Plant and Microbial Biology, North Carolina State University, Raleigh, North Carolina

<sup>2</sup>Department of Cell Biology and Physiology, and Microbiome Core Facility, University of North Carolina School of Medicine, Chapel Hill

### Abstract

Prebiotics are ingredients selectively fermented by the intestinal microbiota that promote changes in the microbial community structure and/or their metabolism, conferring health benefits to the host. Studies show that  $\beta$  (1–4) galacto-oligosaccharides [ $\beta$  (1–4) GOS], lactulose and fructo-oligosaccharides increase intestinal concentration of lactate and short chain fatty acids, and stool frequency and weight, and they decrease fecal concentration of secondary bile acids, fecal pH, and nitroreductase and  $\beta$ -glucuronidase activities suggesting a clear role in colorectal cancer (CRC) prevention. This review summarizes research on prebiotics bioassimilation, specifically  $\beta$  (1–4) GOS, and their potential role in CRC. We also evaluate research that show that the impact of prebiotics on host physiology can be direct or through modulation of the gut intestinal microbiome, specifically the probiome (autochthonous beneficial bacteria), we present studies on a potential role in CRC *progression* to finally describe the current state of  $\beta$  (1–4) GOS generation for industrial production.

## 1. Introduction

### 1.1. Prebiotics: Definition and scope of review

Prebiotics are “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health”<sup>[41]</sup>. The gut bacteria play an active role in the digestion of carbohydrates, a fermentative process that yields short chain fatty acids (SFCA) like acetic, propionic and butyric acids, and various gases like hydrogen, methane and CO<sub>2</sub>. The concept of prebiotics is relatively recent; however, the differences between non-digestible and digestible carbohydrates, and the methods to quantify them were established early in the last century. Digestible carbohydrates were considered those available for digestion and

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\*To whom correspondence should be addressed: Department of Cell Biology and Physiology, 332 Isaac Taylor Hall - Campus Box 7545, School of Medicine, University of North Carolina, Chapel Hill, NC 27599-7545, azcarate@med.unc.edu, Phone: 919 966 9838.

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absorption in the small intestine that rapidly increased blood glucose levels<sup>[83]</sup> (had a high glycemic index) whereas non-digestible carbohydrates were not digested and hence had a low glycemic index. The methods for determination of digestible carbohydrates target the reducing sugars, including sucrose and starch as a measure of the available carbohydrates in food products. The non-digestible carbohydrates are reported as the amount of insoluble residue, corrected for protein and ash (reviewed by McCleary<sup>[84]</sup>).

Prebiotics are short chain oligosaccharides resistant to digestion in the upper gastrointestinal tract; thus, they can reach the colon undigested, to selectively stimulate the growth of the beneficial members of the intestinal the microbiota or probiome<sup>[6]</sup> that carry functional  $\beta$ -galactosidases and/or  $\beta$ -glucosidases. The most recent definition of prebiotics states that “a dietary prebiotic is an ingredient selectively fermented that results in specific changes in the composition and/or activity of the gastrointestinal (GI) microbiota thus conferring benefit(s) upon host health”<sup>[42]</sup>. Three commercially available dietary ingredients: galacto-oligosaccharides (GOS), lactulose, and fructo-oligosaccharides (FOS) have been used as food additives in Japan and Europe. In the United States, the Food and Drug Administration (FDA) has not stated health claims for probiotics or prebiotics but requires a notification of safety when applying for commercialization of a new dietary ingredient. Additionally, the FDA Centers for Biologic Evaluation and Research and Drug Evaluation and Research (CBER and CDER, respectively) published a draft document regarding the need to file an Investigational New Drug application when doing human research<sup>[1]</sup> in which is mentioned that, according to the National Center for Complementary and Alternative Medicine (NCCAM), prebiotics are included in the domain called “biologically based practices”, which “includes, but is not limited to, probiotics, botanicals, animal-derived extracts, vitamins, minerals, fatty acids, amino acids, proteins, whole diets, and functional foods”. Prebiotics commercialized in the US that have submitted notification to be considered as new dietary ingredients (NDI), and have GRAS (generally regarded as safe status) status for use in foods and term infant formulas, include Vivinal GOS® (Friesland Foods Domo®) and Oligomate 55N/55NP (Yakult Pharm. Ind. Co.). Vivinal GOS® is generated using the  $\beta$ -galactosidase from *Bacillus circulans* while Oligomate, which has been used as a food ingredient in Japan for several years, is generated using whole cells of *Sporobolomyces singularis* overexpressing its own  $\beta$ -hexosyl transferase. Figure 1 shows the proportion of components in commercial and a non-commercial, enriched GOS formulation recently developed<sup>[28]</sup>. The commercial  $\beta$  (1–4) galacto-oligosaccharides [ $\beta$  (1–4) GOS] formulations contain approximately 50%  $\beta$ -(1–4) GOS, and residual glucose, lactose and galactose, carbohydrates that may enhance growth of non-probiomic bacteria. The host and the microbial physiological responses to prebiotics, which are poorly digested by endogenous enzymes and fermented by the intestinal microbiota containing functional  $\beta$ -galactosidases and/or  $\beta$ -glucosidases are referred as “prebiotic effects” and have been extensively documented in humans and animals<sup>[55, 75, 94]</sup>.

**1.1.1. Galacto-oligosaccharides (GOS)**—GOS are the most inexpensive alternative often added to infant formulas to mimic the beneficial effects of the oligosaccharides present in human breast milk and is one of the most extensively evaluated prebiotic. They are considered prebiotics as was originally defined by Gibson and Roberfroid<sup>[41]</sup>: they are not

absorbed in the upper part of the gastrointestinal tract, they are specific substrates for one or a group of beneficial bacteria of the probiome (resulting in the modulation of the intestinal microbiota in favor of a healthier composition), and have beneficial systemic effects on the host. Technically, GOS can have  $\alpha$ - or  $\beta$ -configurations by the nature of the glycosidic bonds between the sugar molecules (Table 1). The majority of published scientific articles use the term GOS when referring to  $\beta$  (1–4) GOS, while the abbreviation TOS has been alternatively employed<sup>[31]</sup>.  $\beta$  (1–4) GOS are generally produced by enzymatic transglycosylation using  $\beta$ -galactosidases or  $\beta$ -glucosidases and have a generic formula of  $\beta$  (1–4) [DGalactose]<sub>n</sub>-D-Glucose where n ranges between 3 and 10 sugar moieties<sup>[104]</sup>. Due to their glycosidic bond, they are not metabolized in the small intestine reaching the colon intact, where they serve as substrate for specific members of the microbiota capable of hydrolyzing the galactose-glucose bonds<sup>[106]</sup>. These carbohydrates and the carbohydrate fragments formed from the hydrolysis of the complex polymeric substances are further transformed by the butyrate-producers in the colon (see below a more detailed description of the effect of GOS on the intestinal microbiota). Products of GOS metabolism include SCFAs, lactate, acetate, and gases in proportions depending upon the  $\alpha$ - or  $\beta$ -configuration of the sugars<sup>[31]</sup>. *Bifidobacterium* species are the most studied members of the probiome able to metabolize GOS, FOS, and human milk oligosaccharides (HMOs). Consequently, an increased abundance of bifidobacteria is the most reported effect of GOS and this is termed “bifidogenic effect”<sup>[29, 130]</sup>.

**1.1.1.1. GOS and CRC prevention:** There is a strong genetic component in the development of colorectal adenomas or cancers, which in conjunction with environmental factors including diet and lifestyle have a major impact on risk (reviewed in<sup>[7]</sup>). Lifestyle aspects related to increased risk of CRC include elevated body mass index (BMI), obesity, and low physical activity<sup>[61, 80]</sup>. With regards to diet, elevated risk of CRC has been associated with high consumption of red and processed meat, refined grains, sweets, and alcohol, and a low consumption of fruits and vegetables<sup>[5, 115]</sup>. In this review, we examined the scientific literature and identified a number of factors, consisting of genetic and environmental parameters conducive to CRC, which can potentially be modulated by prebiotics to prevent gathering of the perfect storm. We have not included all chemopreventive agents of CRC like aspirin and other NSAIDs, multivitamins, hormones, and others<sup>[24]</sup>, instead we assessed the literature specifically to identify research studies that suggest a role of GOS in CRC risk and prevention (Figure 2). The following sections list specific parameters with a role in CRC that could be modulated by prebiotics.

**1.1.1.1.1. Bile acids: decreased concentration of secondary bile acids and increased concentration of primary bile acids?:** In the intact intestinal tract, bacterial biotransformations of conjugated bile acids include deconjugation of bile acids (CBAs) to liberate free primary bile acids (cholic acid [CA] and chenodeoxycholic acid [CDCA]), oxidation of hydroxy groups at C-3, C-7 and C-12 with formation of oxo bile acids, and reduction of these oxo groups to either alpha- or beta-configuration<sup>[57]</sup>. Bacterial bile salt hydrolases (BSH, EC 3.5.1.24) are responsible for deconjugation of CBAs. The enzyme hydrolyzes the amide bond and liberate the glycine/taurine moiety from the steroid<sup>[11]</sup>. Quantitatively, the most important bacterial biotransformation is the 7  $\alpha$ -dehydroxylation of

CA and CDCA, to yield the secondary bile acids deoxycholic (DCA) and lithocholic (LCA) acids. A pioneer study by Reddy and collaborators<sup>[118]</sup> showed that the secondary bile acids sodium cholate or sodium chenodeoxycholate increased *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced adenocarcinomas and adenomas in germ-free rats, and mainly adenomas in conventional female F344 rats. A study by the same group reported that patients with colon cancer excreted high levels of fecal secondary bile acids and cholesterol metabolites compared to healthy individuals<sup>[117]</sup>. Likewise, studies have reported that patients with colorectal adenomas had also increased fecal DCA and LCA<sup>[119]</sup> and serum DCA<sup>[10]</sup>. A more recent meta-analysis of 20 studies including a total of 1,226 individuals, aimed to review observational studies that examined the relationship between fecal bile acids and CRC or adenoma, reported that patients with adenomas and CRC had a higher concentration of total bile acids in stools. According to this analysis, patients with CRC (but not adenoma patients) had significantly increased concentrations of CDCA. One study reported markedly higher concentrations of fecal DCA in CRC patients<sup>[119]</sup>, while most of the other studies included in the meta-analysis reported either no differences between CRC and control patients or higher concentrations of DCA in controls<sup>[151]</sup>. DCA excretion in patients with adenoma was significantly higher than controls. Conversely, LCA excretion was significantly higher in CRC patients, but not in adenoma patients<sup>[151]</sup>. An early study on high-risk CRC patients (individuals that had had adenomas removed) showed no significant differences in concentration of total bile acids in stools compared to controls<sup>[96]</sup>. No human studies have investigated the concentration or excretion of bile acids in patients with CRC or adenomas fed GOS. However, healthy men that consumed 15 g/day of either GOS, FOS or inulin excreted lower concentrations of fecal LCA and DCA, although values only reached statistical significance in the FOS and inulin groups for DCA<sup>[158]</sup>.

**1.1.1.1.2. Decreased fecal pH:** Short chain fatty acids (SCFAs) are responsible for the neutralization of bases and the acidification of colon contents (reviewed by Newmark and Lupton<sup>[100]</sup>). Most SCFAs are generated by colon microbial fermentation of undigested dietary carbohydrates. A study investigating the effects of dietary cellulose and GOS on the development of dimethylhydrazine-induced CRC in rats fed low, medium, or high-fat diets showed that the pH in the cecum of animals fed high-GOS diets was 5.8, significantly lower than animals fed cellulose-diets, which had a pH ranging from 6.4 to 6.6<sup>[163]</sup>. According to data reviewed by Newmark and Lupton<sup>[100]</sup>, the pH within the colon luminal environment can affect the composition and metabolic activity of the microbiota, the absorption of luminal components like SCFAs and minerals, the metabolism of drugs and carcinogens, enzymatic reactions such as those catalyzing secondary bile acid formation, and mucosal cell proliferation.

**1.1.1.1.3. Increased SCFAs:** Perhaps the most studied effect of prebiotics consumption is the increase of the major end-products of carbohydrate fermentation, SCFAs in the colon, of which butyrate has been extensively studied for its role in CRC prevention and progression<sup>[51, 71, 134]</sup>, and that have been reported in studies *in vitro*<sup>[140]</sup>, in animal models<sup>[62, 73, 109]</sup>, and in human infants<sup>[35]</sup>. A fairly constant ratio of acetate > propionate > butyrate (in a molar ratio of approximately (60:20:20) has been reported in population surveys and measured in the intestinal contents of victims of sudden death, which however

can be altered by dietary changes<sup>[26, 165]</sup>. The role of SCFAs in the intestine include nutrition of the host colonic epithelium, modulation of the colonic pH, intracellular pH and cell volume, and regulation of proliferation and gene expression<sup>[8, 36, 90, 100]</sup>. Consequences of increased concentration of colonic SCFAs include decreased pH, which influences composition of the microbiota, decreased solubility of bile acids, increased absorption of minerals, and reduced absorption of ammonia (reviewed in <sup>[165]</sup>), all of which have a general correlation with SCFAs' anticarcinogenic properties, particularly attributed to butyrate. A study conducted in 1,2-dimethylhydrazine (DMH)-treated rats fed either wheat bran, guar gum, or oat bran showed significantly fewer tumors in the rats fed wheat bran compared with those fed guar or oat bran, with the lowest tumor mass observed in rats fed wheat bran. These results suggested that fiber associated with high butyrate concentrations in the distal large bowel might be protective against CRC, while soluble fibers that do not raise distal butyrate concentrations may not be protective. It also suggested a relationship between butyrate production *in vivo* and suppression of tumor formation<sup>[86]</sup>.

**1.1.1.1.4. Increased lactate:** Prebiotic consumption leads to a transient increase in lactate and butyrate in the colon and has been deemed of importance for CRC prevention<sup>[40]</sup>. However, utilization of GOS by butyrate generating (butyrogenic) bacteria has not been reported. The proposed mechanism by which prebiotics increase butyrate concentration is by cross-feeding (or syntrophy) between different members of the colon microbiota<sup>[31]</sup>. In fact, GOS has been shown to increase microbial abundance of bifidobacteria and lactobacilli in the colon<sup>[29, 77]</sup>, which can produce acetate, lactate, formate, ethanol, and succinate<sup>[39, 157]</sup>, but have not been stated to produce butyrate. Therefore, the butyrogenic effect of prebiotics requires colonic accumulation of butyrate, which in turn needs a syntrophy (cross-feeding) mutualistic relationship between microbial production of lactate and its biotransformation to butyrate<sup>[12, 33, 132]</sup>. In fact, GOS consumption increased lactic acid concentration in fecal samples of healthy young men from an average of 266 mg/100 g to 379 mg/100 g of dry feces, although differences did not reach statistical significance<sup>[158]</sup>.

**1.1.1.1.5. Increased stool frequency and weight:** Large bowel transit time or frequency of bowel movements is one of the most important factors affecting the structure and function of the colonic microbiota<sup>[78]</sup>. Moreover, low frequency of bowel movements (i.e. increased stool retention times) and reduced fecal weight have been associated with higher CRC risk. The best-documented influence of slow colonic transit is on bile acid metabolism by increasing DCA and raising cholesterol saturation of bile<sup>[72]</sup>. Bulking of the stool accelerates its transit, reducing the exposure time to irritants and carcinogens<sup>[27]</sup>. In an animal study, effects of dietary cellulose and GOS on the development of DMH-induced CRC were assessed in rats fed low-, medium- or high-fat diets<sup>[163]</sup>. In animals fed the high-GOS diet, the cecal content was significantly increased in weight and significantly decreased in pH.

**1.1.1.1.6. Decreased azoreductase, nitroreductase, and  $\beta$ -glucuronidase activities:** Nitroreductases and azoreductases reduce nitro- and azo- components to aromatic amines through highly reactive reactions that generate mutagens and carcinogens while  $\beta$ -glucuronidases hydrolyze glucuronic acid conjugates of heterocyclic amines also yielding

reactive metabolites that can cause damage to colonic mucosal cells<sup>[44]</sup>. In 1976, Goldin and Gorbach reported that the effect of diet on nitroreductase, azoreductase, and  $\beta$ -glucuronidase activities in male rats shifted from a diet with high vegetable and grain content to a diet consisting predominantly of beef<sup>[44]</sup>. The animals fed predominantly beef had significantly higher levels of these activities, which have been implicated in the conversion of procarcinogens into carcinogens<sup>[43, 124]</sup>. Moreover, over expression of nitroreductases has been reported in a *Clostridium paraputrificum* strain isolated from patients with colon cancer compared to clostridia from healthy individuals<sup>[99]</sup>. A research study showed that  $\beta$ -glucuronidase and nitroreductase activities were reduced in rats fed GOS, while  $\beta$ -glucosidase activity was increased<sup>[126]</sup>, and the same trend was observed in humans<sup>[158]</sup>. More research on an actual correlation between these activities and CRC is clearly needed.

**1.1.1.2. GOS and CRC progression:** Although two animal studies have suggested that GOS was protective against the development of colorectal tumors, as demonstrated by an inhibitory effect on tumor incidence, multiplicity and size, regardless of the fat content of the diet <sup>[163, 164]</sup>, studies on GOS and a potential role in CRC *progression* are lacking. One study reported that angiogenesis, the formation of new blood vessels from the preexisting vasculature and an obligatory event for the growth and progression of solid tumors beyond the size limit imposed by simple diffusion for nutrients, was inhibited by sulfated GOS. The study revealed that sulfated GOS with a low degree of polymerization (even pentasaccharides) were potent angiogenesis inhibitors with an influence on fibroblast growth factor FGF-2<sup>[65]</sup>. Of potential interest are the studies aimed to determine if human milk oligo-saccharides (HMOs), which are structurally similar to GOS, had a role in modulation of the neonatal intestinal development and enterocyte function. One study reported that exposure of the intestinal cell lines HT-29, HIEC, and Caco-2 to HMOs significantly inhibited cell proliferation<sup>[67]</sup>. Specifically, neutral oligosaccharides had an anti-proliferative effect, a significant induction of apoptosis, and a minor increase in differentiation, and acidic oligosaccharides showed inhibition of the intestinal cell proliferation via induction of alkaline phosphatase activity. Moreover, HMOs induced growth arrest of intestinal cells by modulation of epidermal growth factor receptor (EGFR) signal pathways and cell cycle-associated gene expression<sup>[66]</sup>.

### 1.1.2. Other prebiotics

**1.1.2.1. Lactulose:** Lactulose (4-0- $\beta$ -d-galactopyranosyl-d-fructofuranose) is an artificial disaccharide composed of fructose and galactose, bonded together by a  $\beta$  (1-4)-glycosidic bond. The production and physiological effects of this disaccharide have been reviewed by Schuster-Wolff-Buhring et al.<sup>[135]</sup> and Panesar et al.<sup>[110]</sup>. Originally generated by intensive heating of lactose, several studies have demonstrated that lactulose induces growth of *Bifidobacterium* both in vitro<sup>[30, 108]</sup> and in vivo<sup>[14, 98, 159]</sup> but reports on the same effect on strains of *Lactobacillus* are mixed, with studies reporting either stimulation of *Lactobacillus*<sup>[127]</sup> or no significant differences in their growth rates<sup>[14, 50]</sup>. Besides the prebiotic, or more specifically, bifidogenic effect, lactulose has been shown to enhance colonic motility which correlates with its traditionally use as laxative in the treatment of constipation<sup>[139, 147, 150]</sup>. Additionally, a lower pH in the colon due to selective fermentation of this compound has been linked to an enhanced intestinal solubility of minerals which

resulted in its improved absorption<sup>[138, 156]</sup>. Similarly ascribed to the lowering of the intestinal pH<sup>[129]</sup> and generation of butyrate, one early study showed that lactulose decreased the pH of rat stools. Thus, rats with low pH stool had significantly fewer colon tumors after injections of 1,2-dimethylhydrazine (DMH) than rats treated with DMH alone<sup>[129]</sup>. Other studies have shown that lactulose decreased the incidence of aberrant crypt foci (ACF) and aberrant crypts generated in azoxymethane (AOM)-treated rats<sup>[23]</sup> and that lactulose at a dietary level of 0.3% significantly decreased the degree of DNA damage induced in cells of the colonic mucosa by DMH in rats harboring human gut microbiota<sup>[125]</sup>.

High concentrations of secondary bile acids in feces, blood, and bile have been linked to cholesterol gallstone disease and colon cancer<sup>[85]</sup>. Consumption of lactulose (12 weeks, 60 g/day) in patients with adenomas led to decreased colonic absorption of secondary bile acids, especially DCA. Other outcomes included decreased intestinal transit and fecal pH and increased stool frequency and weight<sup>[155]</sup>. A study by Roncucci et al.<sup>[121]</sup> reported that lactulose (20 g/day) taken daily was effective in reducing the recurrence rate of colon adenomas. Conversely, lactulose consumption did not affect the rectal mucosal proliferation in individuals with a family history of CRC<sup>[122]</sup>.

**1.1.2.2. Fructo-oligosaccharides (FOS):** FOS are short to medium length chains of  $\beta$ -D fructans in which fructosyl units are bound by a  $\beta$  (2-1) osidic linkage<sup>[120]</sup>.  $\beta$  (2-1) fructans include inulin and FOS, and occur naturally in onion, chicory, garlic, asparagus, banana, artichoke, and other vegetables<sup>[75]</sup>. A study in 6 healthy volunteers given FOS for 11 days demonstrated that 89% of the ingested FOS was not metabolized in the small intestine, and none was excreted in stools, indicating that the portion reaching the colon was completely fermented by the colonic microbiota<sup>[92]</sup>. Unlike lactulose, FOS can be fermented by both *Bifidobacterium* and *Lactobacillus* strains<sup>[64, 87, 123]</sup> and studies have shown that FOS consumption results in increased numbers of *Bifidobacterium*<sup>[17]</sup> and *Lactobacillus*<sup>[131, 148]</sup> species. Interestingly, a relatively long term study in rats fed a basal low-fiber diet or the same diet containing 9 g/100 g of FOS for 2, 8 or 27 weeks showed that the increased concentrations of *Lactobacillus* and lactic acid in the cecum were abolished at 8 weeks; however, cecal SCFA concentration and molar butyrate ratio were higher in rats fed FOS at all-time points<sup>[69]</sup>. The prebiotic effect of FOS is dose dependent<sup>[17]</sup>, also associated with a decrease of fecal pH in some animal models<sup>[13, 22]</sup> (results are inconclusive in humans<sup>[15, 16]</sup>), and increased production of lactic acid and SCFAs<sup>[34, 123]</sup>.

Two animal models: the male Sprague-Dawley rat and *Apc*<sup>Min/+</sup> mouse, which are heterozygous for a non-sense mutation of the *Apc* gene, the murine homologue of APC<sup>[95]</sup>, are most commonly used to study the impact of dietary interventions on CRC. Studies in rats showed that FOS reduced the number of aberrant crypt foci in the colon of DMH-treated male Sprague-Dawley rats fed a 60 g FOS/kg diet for 35 days<sup>[56]</sup>, and inhibited ACF formation and crypt multiplicity in AOM-treated male F344 rats (although at a lesser level than inulin)<sup>[116]</sup>. This end result was also demonstrated in rats but only when FOS was fed in combination with celecoxib (a non-steroidal anti-inflammatory drug)<sup>[20]</sup> or soybeans<sup>[46]</sup>. The alternative animal model currently used for preclinical testing of chemopreventive agents is the *Apc*<sup>Min/+</sup> mouse model, although one major drawback of this mouse model is the occurrence of tumors predominantly in the small intestine and not the colon. Corpet and

Pierre<sup>[25]</sup> pointed out that most of the studied chemopreventive agents have a similar efficacy on large and small intestinal tumors, with specific exceptions that include NSAIDs (more protective in small intestine). Conversely, resveratrol, folic acid, uroguanylin, selenium in broccoli, and FOS have a more prominent protective effect on the colon (reviewed in <sup>[25]</sup>). The FOS results are explained by the fact that FOS is not metabolized in the small intestine but fermented by the microbiota in the colon. Pierre et al.<sup>[112]</sup> reported that short-chain (sc) FOS decreased the number of colon tumors in *Apc*<sup>Min/+</sup> mouse. Moreover, the histopathological examination of large tumors in the colon suggested a delay in the transition from adenomas to carcinomas in animals fed scFOS compared to controls, with adenomas being as numerous as adenocarcinomas in this group, whereas adenocarcinomas predominated in the other diets. The same group reported that scFOS also stimulates the local immune system with up-regulation of IL-15 by scFOS in the colon of *Apc +/Min* mice<sup>[9]</sup>.

FOS impact on CRC studies in humans are scarce and contradictory at best. A study with healthy human volunteers revealed that FOS ingestion (12.5 g/day) led to an increase in fecal bifidobacterial counts and  $\beta$ -fructosidase activity but had no significant effect on fecal total anaerobes, pH, activities of nitroreductase, azoreductase, and  $\beta$ -glucuronidase, and concentrations of bile acids and neutral sterols (metabolic indexes potentially involved in colonic carcinogenesis)<sup>[15]</sup>. In a different study, 94 subjects with small adenomas (<10 mm in diameter), larger adenomas, or no adenomas were fed 5 g of scFOS twice daily for 3 months. The study showed that long-term consumption of the prebiotic significantly increased fecal butyrate levels in patients with colon adenomas to the baseline level of adenoma-free patients. Additionally, scFOS consumption reduced fecal concentration of LCA, a secondary bile acid, in subjects without adenomas<sup>[18]</sup>.

The above discussed research indicates that impact of prebiotics on the gut microbiome and parameters of CRC prevention is not exclusive of GOS. However, research shows that differences exist between the different prebiotics, lactulose with a more pronounced laxative effect and a lesser impact on the gut *Lactobacillus* population.

## 1.2. Modulation of the intestinal microbiota by GOS: Who can metabolize GOS in the intestinal tract? How is GOS metabolized by bacteria?

The vast majority of the intestinal bacteria have predominantly saccharolytic metabolisms; as a consequence, the availability of carbohydrates is almost certainly the most important factor controlling the composition and metabolic activities of the gut microbiota, despite the presence of large numbers of amino acid fermenting bacteria and syntrophic species in the colon. The bifidogenic effect of GOS has been documented in infants consuming formula containing polydextrose and  $\beta$  (1–4) GOS, which showed increased abundance of total *Bifidobacterium* species as well as specifically *B. longum* and *B. infantis*<sup>[133]</sup>, and in infants consuming formula supplemented with  $\beta$  (1–4) GOS and FOS (in a 9:1 ratio)<sup>[52, 128]</sup>, which showed not only increased abundance of bifidobacteria in stools of infants that consumed the prebiotics, but also increased abundance of lactobacilli and decreased numbers of *Clostridium difficile*.



It cannot be assured that GOS will be fermented only by probiomic bacteria in the intestinal tract or that the products of fermentation will not benefit growth or activity of potential pathogens. However, the study by Davis et al.<sup>[29]</sup> done in 18 healthy adult human volunteers, whom were administered  $\beta$  (1–4) GOS during 16 weeks showed that only a few taxa other than bifidobacteria, were impacted by GOS. Statistically significant decreases were observed for the family Bacteroidaceae and the genus *Bacteroides* while abundance of *Coprococcus comes* and *Faecalibacterium prausnitzii* was significantly increased at doses of 5 and 10 g/day. Conversely, a recent study using an in vitro model of the colon, the TIM-2 system<sup>[91]</sup>, and <sup>13</sup>C-labeled GOS showed an increased abundance of several *Bifidobacterium* species being the most affected *B. bifidum* and *B. catenulatum*.  $\beta$  (1–4) GOS increased also abundance of *Lactobacillus* species (specifically *L. gasseri* and *L. salivarius*), and commensal, potentially pathogenic bacteria, including members of the family Enterobacteriaceae and *Klebsiella* species. In contrast, species of *Bacteroides* and *Prevotella* decreased in numbers, as well as *Faecalibacterium prausnitzii* (indicated with its former name, *Fusobacterium prausnitzii* in the publication), and species of *Eubacterium*, *Ruminococcus* and *Lactococcus*<sup>[77]</sup>. The study, which used the pooled fresh stools of 8 healthy Dutch adult volunteers to create a standardized microbiota to use as inoculum for the study, also showed that production of SCFA was higher in the GOS pool, with higher acetate and lower propionate production, and slightly lower levels of butyrate. Additionally, levels of *i*-butyrate, *i*-valerate and ammonia (originated from protein fermentation) were lower in the GOS pool<sup>[77]</sup>.

Metabolism of prebiotics in the colon is influenced by the respective sugar monomers, the degree of polymerization, and the type of glycosidic bonds<sup>[146]</sup>. It is accepted that GOS can resist hydrolysis by salivary and intestinal digestive enzymes but they are sensitive to hydrolysis by bacterial enzymes in the colon<sup>[113]</sup>. Although technically GOS could be hydrolyzed by the human intestinal  $\beta$ -galactosidase, hydrolysis is normally limited due to very weak enzymatic activities<sup>[76, 97]</sup>. Different enzymatic systems are involved in the hydrolysis of  $\beta$ -GOS, generally metabolized by  $\beta$ -galactosidases ( $\beta$ -Gal, EC 3.2.1.23), commonly known as lactases<sup>[21]</sup>, since  $\beta$ -Gal enzymes are also responsible for the hydrolysis of terminal non-reducing  $\beta$ -D-galactose residue of the disaccharide lactose (4-O- $\beta$ -galactopyranosyl-D-glucopyranose). Bacterial  $\beta$ -Gal enzymes are quite ubiquitous. They are present in *Proteobacteria*, *Firmicutes*, *Actinobacteria*, the CFB (*Bacteroidetes-Chlorobi-Fibrobacteres*) group, *Verrucomicrobia*, *Spirochaetes*, *Victivallaceae*, *Thermotogales*, *Chloroflexi*, *Acidobacteriales*, and over 350 species more.  $\beta$ -Gal enzymes are of biotechnological relevance due to their uses to synthesize GOS. In this section of the review, we will discuss how bacteria use  $\beta$ -Gal enzymes for GOS hydrolysis. In *E. coli*,  $\beta$ -Gal enzymes have two catalytic activities. They and hydrolyze lactose to galactose plus glucose, or convert lactose to another disaccharide, allolactose, which in turn induces the *lac* operon<sup>[82]</sup>. Due to the fact that the same structural  $\beta$  (1–4) bonds exist in lactose and GOS, the same enzyme is responsible for their hydrolysis. Lactose and GOS are evolutionarily related and represent the highest proportion of carbohydrates in the breast milk of mammals being the first and most natural source of nutrients for the newborn. Consequently, the most extensively characterized  $\beta$ -Gal enzymes are those from *Bifidobacterium* species. *B. bifidum* and some strains of *B. longum* subsp. *longum* exhibit a dedicated pathway for degrading

type I HMOs, that involves liberation of lacto-N-biose type I (LNB) and galacto-N-biose type I (GNB) from their natural substrates by extracellular enzymes (endo- $\alpha$ -N-acetylogalactosaminidase<sup>[38]</sup> and/or lacto-N-biosidase<sup>[162]</sup>), transport and subsequent cleavage by the lacto-N-biose phosphorylase LnpA. The products of this process are  $\alpha$ -galactosylphosphate, which enters glycolysis, and N-acetylhexosamines, which enter the aminosugar metabolic cycle<sup>[105]</sup>. Strains of *B. longum* subsp. *infantis* characterized so far have shown no presence of lacto-N-biosidase homologs<sup>[74]</sup>. A recent study by Yoshida et al.<sup>[166]</sup> showed that *B. longum* subsp. *infantis* can directly incorporate lacto-N-tetraose (LNT) and hydrolyze it *via* a specific  $\beta$ -Gal enzyme. The authors identified two different  $\beta$ -Gal enzymes (Bga42A and Bga2A) responsible for the degradation of type-1 and type-2 HMOs respectively.

$\beta$ -Galactosidases have been isolated and characterized from a number of *Lactobacillus* species, including *L. pentosus*<sup>[79]</sup>, *L. sakei*<sup>[59]</sup>, *L. delbrueckii* subsp. *bulgaricus*<sup>[103]</sup>, *L. plantarum*<sup>[60]</sup>, *L. reuteri*<sup>[102]</sup> and *L. acidophilus*<sup>[101]</sup>. However, the dedicated pathways and enzymes involved in GOS degradation have not been extensively characterized. A transcriptional analysis of genes induced in GOS-supplemented medium showed that in *L. acidophilus*, GOS specifically induced a cluster of genes encoding intracellular proteins involved with galactose and lactose metabolism, notably the LacS permease implicated in GOS transport<sup>[3]</sup>.  $\beta$  (1–4) GOS are structurally similar to oligosaccharides present in mammals breast milk and can be synthetically produced from lactose by the transglycosylating activity of  $\beta$ -galactosidases (discussed above) while  $\alpha$ -GOS can be found in natural reservoirs such as soybeans<sup>[89]</sup>.  $\alpha$ -GOS (galactosyl-sucrose oligosaccharides) include raffinose, stachyose and verbascose and consist of galactose residues linked  $\alpha$  (1–6) to the glucose moiety of sucrose (Table 1). Given that most mammals do not express pancreatic  $\alpha$ -Galactosidases ( $\alpha$  (1–6) Gal), its digestion is mediated by colonic bacterial enzymes. The fermentation of  $\alpha$  (1–6) GOS results in fermentative gases, which can induce abdominal pain and flatulence<sup>[114, 144]</sup>. Bacterial  $\alpha$  (1–6) Galactosidases (EC 3.2.1.22) are clustered into families 4, 27 and 36 of the sequence-based classification of glycoside hydrolases (GH)<sup>[48, 49]</sup>. A search for EC 3.2.1.22 in the Kyoto Encyclopedia of Genes and Genomes (KEGG)<sup>[63]</sup> retrieved 1,114 hits, including genes from *Bifidobacterium* and *Lactobacillus*, indicating that  $\alpha$ -Gal hydrolases are widely distributed in microbial communities.  $\alpha$ -Gal enzymes have been identified and characterized in several microorganisms including *L. plantarum*<sup>[143]</sup>, *L. reuteri*<sup>[153]</sup>, *Thermotoga neapolitana*<sup>[32]</sup>, *Bacillus stearothermophilus*<sup>[149]</sup>, *Enterococcus faecium*<sup>[168]</sup>, *Enterobacter cloacae*<sup>[107]</sup>, *Citrobacter freundii*<sup>[141]</sup>, species of *Bifidobacterium*<sup>[53, 169]</sup> and recently, *Ruminococcus gnavus*, a human intestinal isolate<sup>[2]</sup>. The *R. gnavus*  $\alpha$ -Gal is encoded by the *agal* gene, which is 2.2 kb in size, monocistronic, and predicted to encode a 743-amino acid (aa) protein, with a 41% similarity to the  $\alpha$ -Gal encoded by *Thermotoga*<sup>[2]</sup>. In *L. plantarum* ATCC 8014,  $\alpha$ -Gal is encoded by the *melA* gene. The enzyme has a predicted 738 aa-size and a molecular mass of 84 kDa. Although *melA* is flanked by two terminators, it is immersed in a gene cluster involved in utilization of  $\alpha$ - and  $\beta$ -galactosidases, including *rafP*, a putative raffinose transporter. No sorting signals were identified in the protein encoded by *melA* suggesting its presence as a soluble enzyme in the cytoplasm of *L. plantarum*<sup>[143]</sup>. According to the KEGG database,  $\alpha$ -Gal hydrolyzes terminal, non-reducing  $\alpha$ -D-galactose

residues in  $\alpha$ -D-galactosides, including GOS, galactomannans, galactolipids, and also hydrolyzes  $\alpha$ -D-fucosides<sup>[145]</sup>. A protein search at NCBI (<http://www.ncbi.nlm.nih.gov/guide/proteins/>) performed in June of 2014, showed 22,588 bacterial entries for  $\alpha$ -Gal enzymes, with 6,632 representatives in the Firmicutes phylum (almost 50% of those corresponding to the order Lactobacillales), 7,430 entries in the Proteobacteria (mostly gamma-Proteobacteria), and 4,797 entries in the phylum Actinobacteria (mostly Actinomycetales).

### 1.3. GOS effects on host physiology and immune system

Numerous research studies have shown that prebiotics have an effect on the intestinal microbiota (discussed above). The anaerobic breakdown of GOS and other prebiotics increase the concentration of fermentation products like lactate and SCFAs, which per se affect the intestinal physiology. The purpose of this section is to summarize available information concerning a direct effect of GOS on the intestinal cells and physiology.

A potential effect of GOS on the intestinal mucus layer has been described in a number of studies; however, the molecular mechanisms involved have not been fully elucidated. In the colon, the mucus layer is a bilayered system. The outer layer, which can be removed by suction, appears to be vital in reducing shear stress to the mucosa<sup>[4]</sup>, and can also trap bacterial pathogens. The underlying adherent layer, which cannot be removed by suction, is normally free of bacteria and may act as a size exclusion barrier to prevent translocation of damaging luminal agents<sup>[19, 47]</sup>. Brownlee et al.<sup>[19]</sup> showed that rats consuming a diet containing cellulose or a fiber-deficient diet had significantly lower colonic resting mucus thickness. Conversely, rats fed ispaghula (a plant of the genus *Plantago* commonly used as a bulk-forming laxative) had a significantly thicker adherent layer and higher total mucus secretion. The authors hypothesized that there could be a direct molecular interaction between specific dietary fibers and the colonic mucin. Moreover, complex carbohydrates from fiber could be absorbed through colonic antigen-presenting cells resulting in stimulation of mucus production. A study by Zhong et al. (2009) showed that GOS feeding significantly improved intestinal barrier function in rats with severe acute pancreatitis<sup>[170]</sup>. An earlier study by Meslin et al.<sup>[88]</sup> showed that consumption of  $\beta$  (1–4) GOS [(referred to as trans galacto-oligosaccharides (TOS) in the study)] modified the mucin cell distribution in the colon of germ-free rats. The authors hypothesized that the lower number of mucus cells observed for the three types of mucins (neutral, acid, and sulphonated) in the proximal colon and the greater number in the distal colon was related to changes in osmolality produced by  $\beta$  (1–4) GOS. Conversely,  $\beta$  (1–4) GOS had no effect in the colon of conventional animals, but reduced the number of acid mucin-containing cells in the cecum, maybe due to higher concentrations of SCFA in the cecum<sup>[88]</sup>. Interestingly, a study on the small intestine of BALB/c mice showed that protein content of the mucosa of  $\beta$  (1–4) GOS-consuming mice was higher than controls, and  $\beta$  (1–4) GOS ingestion also increased significantly the mucin content compared to controls. However, expression of MUC-2 and MUC-4 encoding the major mucins found in the colon, showed no significant differences between  $\beta$  (1–4) GOS-consuming mice and controls<sup>[70]</sup>, contrarily to a reported increased MUC-3 expression induced by inulin feeding in three-week old Sprague–Dawley rats<sup>[111]</sup>. Taken together, these

findings suggest that  $\beta$  (1–4) GOS might stimulate mucus production; however, the mechanisms involved in such stimulation are not well understood.

The gastrointestinal tract hosts a major part of the body's immune system: the gut-associated lymphoid tissue. Few reports have studied interactions between GOS and the mucosal immune system of the gut, and, given their effect on the intestinal microbiota, it is not easy to elucidate whether prebiotics exert direct or indirect modulatory effects. A recent study investigated the efficacy of  $\beta$  (1–4) GOS on colitis development in *Smad3*<sup>-/-</sup> mice after infection with *Helicobacter hepaticus*<sup>[45]</sup>. The study found that  $\beta$  (1–4) GOS reduced the severity of colitis, augmented NK cell function and IL-15 production. This was associated with 1.5-fold increase in fecal bifidobacteria. In a different study, Vaisman et al.<sup>[154]</sup> investigated the effect of a mixture of FOS,  $\beta$  (1–4) GOS, and acidic oligosaccharides on the number and consistency of stools and on immune system biomarkers in children age 9 to 24 months with acute diarrhea. No significant effects were observed on stool characteristics over the first 10 days of treatment, or cytokine profiles during the first 2–3 days of treatment. In fact, a large variability in magnitude and direction of cytokine secretion was detected in patients included in the study. However, when the authors further divided each group dichotomously according to the percentage of subjects who had an increase, no change or a decrease in the levels of different cytokines over the first 2–3 days of treatment, they observed that levels of TNF- $\alpha$  decreased significantly more in the prebiotics supplemented group. Finally, a study investigated the effects of  $\beta$  (1–4) GOS as potential modulators of the elderly intestinal microbiota and their immune system<sup>[161]</sup>. In the double-blind, placebo-controlled, crossover study, the authors detected significant increases in phagocytosis, NK cell activity, and IL-10 production and a significant reduction of proinflammatory cytokines (IL-6, IL-1 $\beta$ , and tumor necrosis factor- $\alpha$ ). It is clear from the limited number of research studies that more studies in animal models as well as in humans are needed to demonstrate an influence of GOS on the immune system. Especially considering that a number of studies suggest a beneficial effect of FOS<sup>[9, 37, 54, 167]</sup> and HMOs (reviewed by Vos et al.<sup>[160]</sup>).

In addition to the impact on the gut microbiome and the host immune system, prebiotics may exert beneficial effects by directly inhibiting the adherence of pathogens to the host epithelial cell surface. The proposed mechanism of action is based on the observation that the structure of free oligosaccharides and glycoproteins of human breast milk can resemble pathogen receptors on intestinal epithelial cells and may act as anti-adhesive molecules that competitively inhibit bacterial adherence<sup>[68]</sup>. In vitro studies showed that GOS significantly inhibited adhesion of microcolony-forming enteropathogenic *Escherichia coli* E2348/69 on HEp-2 and Caco-2 cells in a dose dependent fashion<sup>[142]</sup> and reduced the invasion and adherence of *Salmonella* Typhimurium in three dimensionally cultured HT-29-16E cells<sup>[136]</sup>. Moreover, a research study showed that GOS low molecular weight fractions (primarily tri and tetra-saccharides) in BiMuno® significantly stimulated both pro- and anti-inflammatory cytokines in vitro, a mechanism that may contribute to a reduction in *Salmonella* Typhimurium colonization ability<sup>[137]</sup>. Additionally, dietary GOS supplementation in neonates in combination with *B. breve* and *B. vulgatus* may be an important factor for suppressing growth of *C. perfringens*<sup>[93]</sup>.

#### 1.4. Enzymatic synthesis of $\beta$ (1–4) GOS

For a long time, lactose was considered an inconvenient sub product of the dairy industry and a health issue in dairy products due to lactose intolerance in a growing number of individuals. Therefore, lactose hydrolysis to glucose and galactose using  $\beta$ -galactosidases was a common practice until the beneficial impact of GOS on GI health was acknowledged and methods to recycle lactose into a functional food ingredient [ $\beta$  (1–4) GOS] were envisioned. Prior to that GOS generation during this enzymatic process was usually observed and considered detrimental albeit low accumulation of GOS normally occurs during commercial fermented food preparations<sup>[81]</sup>. Multiple  $\beta$ -galactosidases have been evaluated to improve GOS synthetic activity; however, since they are predominantly hydrolytic enzymes, their efficiency to generate pure GOS is generally limited. Several reactor designs and operational processes have been designed to improve GOS synthesis efficiency<sup>[58, 152]</sup>, but lactose conversion into  $\beta$  (1–4) GOS never exceeds 50%. Such is the case for the  $\beta$ -galactosidase catalyzing the generation of Vivinal GOS® or the process using *Sporobolomyces singularis* membrane bound  $\beta$ -hexosyl transferase to catalyze the synthesis of Oligomate 55N/55NP (Figure 1). In the final formulation of these products there are significant concentrations of lactose, glucose, and galactose (sugars that can impact the physiological influence of the prebiotic) and the final  $\beta$  (1–4) GOS composition and purity vary due to fundamental process differences. Therefore, the traditional options are  $\beta$ -galactosidases (glycosyl-hydrolases or galactosydases) versus  $\beta$ -hexosyl transferases (glycosyl-transferases or glucosydases). The soluble  $\beta$ -hexosyl transferase from *Sporobolomyces singularis* permits a more controllable process able to reach values of 95%  $\beta$  (1–4) GOS with a narrower spectrum of oligosaccharides (mostly  $\beta$  (1–4) GOS-3 and  $\beta$  (1–4) GOS-4) and only a residual small proportion of lactose<sup>[28]</sup>.

#### 1.5. Conclusion

Prebiotics have been consumed by humans for millennia. Moreover, the general health benefits associated with prebiotic consumption have been clearly recognized in the past century. Nonetheless, it is now, with the advent of next generation sequencing technology and bioinformatics tools that researchers have the opportunity to dissect dietary effects and identify key bacterial players, components of the gut microbiota, influencing fermentation and generation of secondary metabolites. This review presented research studies on GOS and CRC that clearly suggest a potential role in prevention. Moreover, this role is supported by a randomized, double-blind, diet-controlled clinical trial that demonstrated that GOS significantly increased fecal acetate and decreased DCA and beta-glucuronidase activity in healthy adult men<sup>[158]</sup>. A clear association between GOS and CRC progression however remains to be elucidated.

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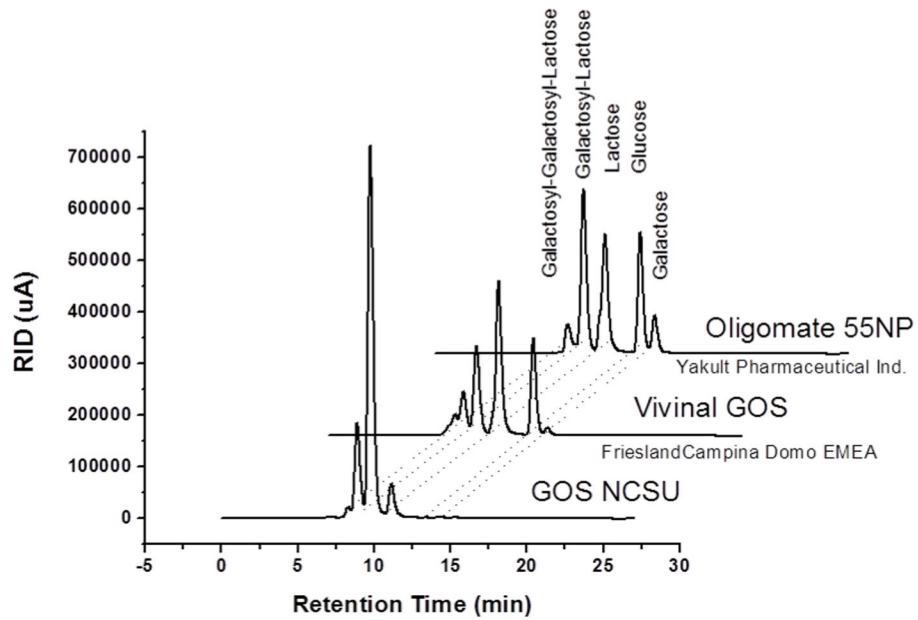
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### Highlights

- $\beta$  (1–4) galacto-oligosaccharides [ $\beta$  (1–4) GOS], lactulose and fructo-oligosaccharides are prebiotics that have clear role in colorectal cancer (CRC) prevention.
- $\beta$  (1–4) GOS increase intestinal concentration of lactate and short chain fatty acids as well as stool frequency and weight, and they decrease fecal concentration of the secondary bile acid lithocholic acid, fecal pH, and nitroreductase and  $\beta$ -glucuronidase activities.
- This review summarizes the current state of research on the bioassimilation of prebiotics through modulation of the intestinal microbiota, and the impact of prebiotics on host physiology and immune system.
- We also present studies on a potential role in CRC *progression* and briefly describe the current state of  $\beta$  (1–4) GOS enzymatic synthesis for industrial production.





**Figure 1.** Purity comparison of residual sugars (lactose, galactose and glucose) and  $\beta$  (1–4) GOS (Gos-3, Gos-4, Gos-5) present in GOS NCSU, Oligomate 55NP (Yakult), Vivinal GOS (Friesland Campina Dome). The enzymes used to generate these products were  $\beta$ -hexosyl-transferase (GOS NCSU and Oligomate 55NP) and  $\beta$ -galactosidase (Vivinal GOS).

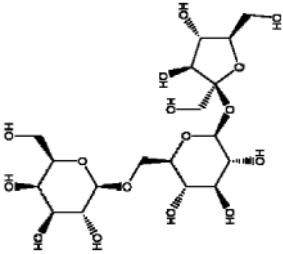
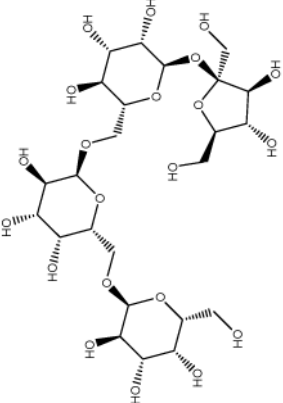
CRC PREVENTION
<ul style="list-style-type: none"><li>• Decrease lithocholic acids (LCA) in stools</li><li>• Decrease fecal pH</li><li>• Increase colonic short chain fatty acids (SCFAs)</li><li>• Increase colonic lactate</li><li>• Increase stool frequency and weight</li><li>• Decrease azoreductase, nitroreductase, and beta-glucuronidase activities</li></ul>
CRC PROGRESSION
<ul style="list-style-type: none"><li>• Inhibit angiogenesis?</li><li>• Anti proliferative effect?</li><li>• Induce apoptosis?</li><li>• Increase differentiation?</li></ul>

**Figure 2.** Summary of the potential impact of  $\beta$  (1–4) GOS on CRC prevention and progression according to published scientific literature.

Table 1

Chemical structure, synthesis methods, and enzymes required for biotransformation of different oligosaccharides.

	Oligosaccharide	Chemical Structure	Glycosidic Linkages	Synthesis	Enzymes required for biotransformation
<b><math>\beta</math> (1-4) GOS</b>	GalactosylLactose		$\beta$ (1,4) glycosidic linkages	Biological	$\beta$ -Galactosidases and/or $\beta$ -Glucosidases
<b>Lactulose</b>	Lactulose		$\beta$ (1,4) glycosidic linkages	Chemical	$\beta$ -Galactosidases
<b>FOS</b>	Fructo-oligosaccharides		$\alpha$ (1,2) glycosidic linkages	Biological	Fructosidase

	Oligosaccharide	Chemical Structure	Glicosidic Linkages	Synthesis	Enzymes required for biotransformation
$\alpha$ (1-6) GOS	Galactosyl-sucrose Raffinose	 <p>The structure shows a sucrose molecule (galactose linked to glucose) with a galactose unit attached to the glucose part of the sucrose via an <math>\alpha</math>(1-6) glycosidic linkage.</p>	$\alpha$ (1,2) and $\alpha$ (1,6) glycosidic linkages	Biological	Fructosidase and $\alpha$ -Galactosidases
	Galactosyl-sucrose Stachyose	 <p>The structure shows a sucrose molecule with two galactose units attached to the glucose part of the sucrose via <math>\alpha</math>(1-6) glycosidic linkages, forming a stachyose derivative.</p>	$\alpha$ (1,2) and $\alpha$ (1,6) glycosidic linkages	Biological	Fructosidase and $\alpha$ -Galactosidases