HAROKOPIO UNIVERSITY

DEPARTMENT OF NUTRITION AND DIETETICS

PhD THESIS

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"Impact of beta-glucan on the intestinal microbiota of a high risk colon cancer population (polypectomised patients)"

ATHENS 2016

Acknowledgements

I would like to thank the hospital staff from the 2nd Department of Gastroenterology, Evangelismos Hospital, Athens and from the Department of Gastroenterology, "Saint Panteleimon" General Hospital, Nikea, as well as **Dr. Stavros S. Tsiakos**, for providing help in subject recruitment. I am especially grateful to **Dr. Dimitrios Karamanolis** and **Dr. John K. Triantafillidis**, for helping me to better understand CRC disease.

I am thankful to Jotis S.A Food Industry for providing both the control and β-glucan enriched flour in order to make the placebo and the experimental food.

I am grateful to **Dr. Panajotis Georgiadis, Mrs. Margarita Bekyrou**, and **Ms. Christiana Fragkouli** for their assistance with comet assay, and **Mrs. Irini Kolonti** for her outstanding technical assistance in Caco-2 cell culture.

I am grateful to **Dr. Tzortzis Nomikos** for the first-rate knowledge and assistance with enzyme testing and to **Dr. Kostas Mountzouris** for the excellent aid he provided with SCFA determination.

I express thanks to all my girls, **Ms.Sissi Tsouvelakidou**, **Ms.Maria Kassara** and **Ms.Lydia Chassioti**, students at the Dept. of Nutrition & Dietetics, who helped me with subject recruitment, sample collection and handling, and bread distribution. I would also like to thank **Mrs.Iota Lianea** for the help she provided in the preparations of the materials and plates on numerous days I was running late to bring in samples. Most of all, thanks to **Ms. Evdokia Mitsou** for excellent assistance in laboratory techniques and in results statistics and for being a good friend through all these years.

And last but not least, I thank my supervisors, **Dr. Adamantini Kyriacouand Dr. Vasiliki Pletsa** for sharing their knowledge on the subject and research, for the support and understanding they have shown towards me during our time together.

Yours truly,

Katja T. Turunen

Athens 22.7.2016

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Content

Summary

Introduction: Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide. The development of CRC is a long, multi-stage process that starts with precancerous tumours (polyps) and involves an interaction between dietary factors, colonic epithelium, and intestinal microbiota. Microbiota could promote CRC development by inducing a chronic inflammatory state, through the biosynthesis of genotoxins interfering with the cell cycle regulation or directly damaging DNA, through the production of toxic metabolites, and by converting dietary heterocyclic amines to pro-carcinogenic compounds. Cereal β-glucans are polysaccharides occurring in the bran of cereal grains that exert immune-modulating and antitumor activity. However, the data from human studies is limited. The aim of this study was to investigate the effect of barley-derived β-glucan on the gut microbiota of polypectomised patients and to examine whether β-glucan consumption by polypectomised patients affects their faecal water (FW) genotoxicity and cytotoxicity*in vitro*.

Methods: Polypectomised men and women who had histologically confirmed neoplastic polyps (≥1cm in size), or hyperplastic polyps (≥3 in number), were randomly assigned to consume 125g of bread per day supplemented with barley β-glucan (3g/d), or without (placebo group), for three months. Stool samples were collected at the beginning of the study (t=0), on the 30th day, on the 90th day and two weeks after the intervention, for the enumeration of the total mesophilic aerobic and anaerobic faecal microbiota, *Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* spp., *Bacteroides* spp., *Enterococcus* spp., total coliforms and *Escherichia coli.*, and *Candid*a spp, using plate count techniques. Faecal bacterial enzyme activity (β-glucuronidase and β-glucosidase), pH, faecal moisture, and the volatile fatty acids were also measured. Subjects' faecal water (FW) was collected and its cytotoxicity and genotoxicity was estimated on Caco-2 cells *in vitro*, using trypan blue exclusion test and COMET assay, respectively. The subjects' gastrointestinal symptoms, physical and mental health states, and physical activity levels were recorded. A nutritional analysis was also done via a 3-day food diary.

Results: Sixty nine polypectomised men and women were recruited into the study (mean age 61.1 years), but only 57 completed. From 57 volunteers according to the randomized design of the study, 27 volunteers (16 men and 11 women) consumed the placebo and 30 volunteers (13 men and 17 women) the experimental bread. A sufficient volume of faecal water was collected from 54 subjects. Overall no significant changes were recorded in the bacterial microbiota between the two feeding groups during the three months ingestion of barley-derived β-glucan.

Bifidobacteriacounts had a trend to increase during the intervention. Group specific analysis for bifidobacteria in the β-glucan group showed an increase at the end of the study (90th day) compared to the baseline. However, bifidobacteria were also found increased in the control group at the end of the study compared to the baseline and to the 30th day. A trend for increase was noted in the *Lactobacillus* spp. count in the β-glucan group on the 90th day of the study compared to the 30th day. In addition, in the β-glucan group we noticed a decrease in enterococci, two weeks after the intervention compared to the 90th day.Also, in the control group enterococci decreased on the 90th day, and 2 weeks after the intervention compared to the baseline. From SCFAs measured, an increase was recorded in the valerate concentration and in the sum of other SCFA (valerate + caproate + heptanoate) concentrations on the 90th day compared to the control group. Butyrate concentration in the control group increased at the end of the intervention compared to the β-glucan group. Among the baseline faecal water samples 49% showed genotoxicity. Genotoxicity in the intervention group decreased during the trial reaching statistical significance on the 90th day compared to the control. An increase was noticed two weeks after the trial. However, it still remained significantly lower comparedto the control.Group specific analysis for β-glucan also revealed a significant decrease in the genotoxicity on the 90th day compared to the baseline. Except for the increase in the flatulence score at the end of the intervention (Day 90-97), we did not notice other significant differences in the gastrointestinal symptoms scores between the feeding groups. Group specific analysis for the β-glucan group revealed a significantly decreased number of evacuations at the end of the intervention (Day 90-97) compared to the baseline.

Conclusions: Barley β-glucan ingestion in polypectomised patients was practically well tolerated. It resulted in an increase in bifidobacteriacounts. However, bifidobacteria were also found increased in the control group. A trend for increase was also noticed in lactobacilli levels. Valerate and the sum of other SCFA concentrations increased during the intervention. The three month administration of barley β-glucanalso significantly decreased the FW genotoxicity.

Key words: Colorectal cancer (CRC), β-glucan, intestinal microbiota, faecal water (WF)

Περίληψη

Εισαγωγή: Ο καρκίνος του παχέος εντέρου (CRC) είναι ο τρίτος σε συχνότητα εμφάνισης καρκίνος στους άνδρες και δεύτερος στις γυναίκες παγκοσμίως. Η διαδικασία της καρκινογένεσης ξεκινά από την εμφάνιση προ-καρκινικών όγκων (πολύποδες), είναι μακρόχρονη και περιλαμβάνει αρκετά ενδιάμεσα στάδια,. Η διατροφή είναι από τους σημαντικότερους περιβαλλοντικούς παράγοντες που διαμορφώνουν τη σύσταση του εντερικού μικροβιόκοσμου. Θεωρείται ότι υπάρχει αλληλεπίδραση μεταξύ της διατροφής, του εντερικού μικροβιόκοσμου και του εντερικού επιθηλίου του ξενιστή. Οι β-γλυκάνες από δημητριακά είναι πολυσακχαρίτες που εμφανίζονται στο πίτουρο των σπόρων των δημητριακών. Μεταξύ των ευεργετικών δράσεων στην υγεία του ανθρώπου, ασκούν ανοσορυθμιστική και αντικαρκινική δράση, όμως τα στοιχεία από έρευνες σε ανθρώπους είναι περιορισμένα.

Σκοπός: η διερεύνηση α) η επίδραση της β-γλυκάνης που προέρχεται από κριθάρι στον εντερικό μικροβιόκοσμο ασθενών που έχουν υποβληθεί σε πολυποδεκτομή και β) να εξεταστεί εάν η κατανάλωση της β-γλυκάνης από τους ασθενείς αυτούς επιδρά στη γενοτοξικότητα και κυτταροτοξικότητα της υδατικής φάσης των κοπράνων τους, σε επιθηλιακά κύτταρα του εντέρου, *invitro*.

Μεθοδολογία: Οι εθελοντές που πήραν μέρος στην κλινική μελέτη ήταν ασθενείς με πολύποδες και κατατάχθηκαν τυχαία σε δύο ομάδες: την ομάδα που κατανάλωνε το πειραματικό τρόφιμο, ψωμί (125g/ημερησίως) εμπλουτισμένο με β-γλυκάνη (3g) ή την ομάδα που κατανάλωνε την ίδια ποσότητα ψωμιού που δεν είχε εμπλουτιστεί (εικονικό τρόφιμο) για τρεις μήνες. Από τους εθελοντές συλλέχθηκαν δείγματα κοπράνων κατά την έναρξη (t=0), την $30^{η}$ ημέρα, την $90^{η}$ ημέρα και δύο εβδομάδες μετά τη λήξη της παρέμβασης. Καταμετρήθηκαν οι ολικοί μεσόφιλοι αερόβιοι και αναερόβιοι μικροοργανισμοί, τα βακτήρια από τα γένη *Lactobacillus, Bifidobacterium, Clostridium, Bacteroides, Enterococcus*, ολικά coliforms και *Escherichiacoli,* καθώς επίσης και η ζύμη*Candida*spp, χρησιμοποιώντας καλλιεργητικές τεχνικές. Μετρήθηκαν επίσης, η δραστικότητα δύο βακτηριακών ενζύμων των κοπράνων, της β-glucuronidase και β-glucosidase, το pH, η υγρασία των κοπράνων και τα πτητικά λιπαρά οξέα. Η κυτταροτοξικότητα και η γονοτοξικότητα της υδατικής φάσης των κοπράνων εκτιμήθηκαν σε επιθηλιακά εντερικά κύτταρα (Caco-2), με τη χρήση της χρωστικής trypanblue και με τη μέθοδο COMET αντίστοιχα. Επίσης καταγράφηκαν η γαστρεντερική συμπτωματολογία των ατόμων, η φυσική και ψυχική κατάσταση της υγείας τους καθώς και η φυσική τους δραστηριότητα, ενώ

επίσης έγινε εκτίμηση της διαιτητικής τους πρόσληψης μέσω τριήμερου ημερολογίου πρόσληψης τροφίμων.

Αποτελέσματα: 69 άνδρες και γυναίκες που είχαν υποβληθεί σε πολυποδεκτομή συμμετείχαν στη μελέτη (μέση ηλικία 61,1), ενώ 57 ολοκλήρωσαν. Από αυτούς, 27 εθελοντές (16 άνδρες και 11 γυναίκες) κατανάλωσαν το υποκατάστατο και 30 εθελοντές (13 άνδρες και 17 γυναίκες) το πειραματικό ψωμί. Ικανοποιητική ποσότητα από την υδατική φάση των κοπράνων συλλέχθηκε από 54 εθελοντές.

Ο πληθυσμός του *Bifidobacterium*spp. εμφάνισε αυξητική τάση κατά τη διάρκεια της παρέμβασης. Η επιμέρους ανάλυση της ομάδας που κατανάλωσε β-γλυκάνη ως προς τον πληθυσμό του *Bifidobacterium*spp., έδειξε ότι είναι σημαντικά υψηλότερος την $90^{η}$ ημέρα σε σύγκριση με την έναρξη. Όμως, το *Bifidobacterium*spp. αυξήθηκε παράλληλα και στην ομάδα ελέγχου. Τάση για αύξηση παρατηρήθηκε στο *Lactobacillus*spp. στην ομάδα της β-γλυκάνης την $90^{η}$ ημέρα της μελέτης σε σχέση με την $30^{η}$ ημέρα. Επιπρόσθετα, ο πληθυσμός των εντερόκοκκων μειώθηκε σημαντικά δύο εβδομάδες μετά την παρέμβαση σε σχέση με την $90^{η}$ ημέρα.Επίσης,στην ομάδα ελέγχου οι εντερόκοκκοι μειώθηκαν την $90^{η}$ ημέρα καθώς και δύο εβδομάδες μετά την παρέμβαση σε σχέση με την έναρξη.

Σχετικά με τα λιπαρά οξέα μικρής αλυσίδας (SCFAs) που μετρήθηκαν, παρατηρήθηκε αύξηση στην συγκέντρωση του βαλερικού οξέος καθώς και στο άθροισμα των άλλων SCFAs (βαλερικό + καπροϊκό + επτανοϊκό) κατά την $90^{η}$ ημέρα συγκρινόμενα με την ομάδα ελέγχου. Η συγκέντρωση του βουτυρικού οξέος αυξήθηκε στην ομάδα ελέγχου σε σχέση με την ομάδα β-γλυκάνης.

Γενοτοξικότητα παρουσίασαν 49% των δειγμάτων της υδατικής φάσης των κοπράνων, κατά την έναρξη της παρέμβασης. Η γενοτοξικότητα στην ομάδα της β-γλυκάνης μειώθηκε κατά τη διάρκεια της παρέμβασης φτάνοντας σε στατιστικά σημαντικά επίπεδα την $90^{η}$ ημέρα σε σύγκριση με την ομάδα ελέγχου. Δύο εβδομάδες μετά την παρέμβαση παρατηρήθηκε μία αύξηση, παρόλα αυτά παρέμεινε ακόμη σε σημαντικά χαμηλότερα επίπεδα σε σχέση με την ομάδα ελέγχου. Η επιμέρους ανάλυση για την ομάδα της β-γλυκάνης έδειξε επίσης μείωση της γενοτοξικότητας την $90^{η}$ ημέρα σε σχέση με την έναρξη.

Με εξαίρεση την αύξηση του μετεωρισμού προς το τέλος της παρέμβασης (ημέρα 90-97) στην ομάδα της β-γλυκάνης ως προς την ομάδα ελέγχου, δεν παρατηρήθηκαν άλλες σημαντικές διαφορές στα γαστρεντερικά συμπτώματα μεταξύ των δύο ομάδων. Η επιμέρους ανάλυση της ομάδας της β-γλυκάνης έδειξε σημαντική μείωση στον αριθμό των κενώσεων προς το τέλος της παρέμβασης (ημέρα 90-97) σε σύγκριση με την έναρξη.

Συμπεράσματα:

- Οι εθελοντές κατανάλωσαν το εμπλουτισμένο ψωμί με β-γλυκάνη για τρεις μήνες χωρίς κάποια σημαντικά γαστρεντερολογικά προβλήματα
- Μειώθηκε σημαντικά η γενοτοξικότητα της υδατικής φάσης των κοπράνων των εθελοντών, σε σχέση με εκείνους που κατανάλωσαν το εικονικό τρόφιμο.
- Παρατηρήθηκε αύξηση των βακτηρίων του γένους *Bifidobacterium*, ενώ παράλληλα σημειώθηκε αύξηση των ίδιων βακτηρίων και στην ομάδα ελέγχου. Τάση για αύξηση στην ομάδα της β-γλυκάνης παρατηρήθηκε επίσης και στα επίπεδα του γένους *Lactobacillus.*
- Σχετικά με τα SCFAs, παρατηρήθηκε αύξηση στο βαλερικό οξύ και στο άθροισμα των συγκεντρώσεων των υπολοίπων SCFAsκατά τη διάρκεια της παρέμβασης.

Λέξεις κλειδιά: Καρκίνος του παχέος εντέρου (CRC), β-γλυκάνη, εντερικός μικροβιόκοσμος, υδατική φάση των κοπράνων (WF)

Abbreviations

ACF = Aberrant Crypt Foci

BMI = Body Mass Index

Caco-2 = Human epithelial colorectal adenocarcinoma cells

CFU = Colony Forming Units

COMET-assay = Single Cell Gel Electrophoresis

CRC = Colorectal Cancer

DMH = 1,2-Dimethylhydrazine

DNA = Deoxyribonucleic Acid

FW = Faecal Water

GI = Gastro Intestinal

GOS = Galactooligosaccharides

HCA = Carcinogenic Heterocyclic Amines

HCT-116 = Human colon carcinoma cells

HT-29 = Human colon adenocarcinoma cells

IFN-γ = Interferon gamma

IL-10 = Interleukin 10

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

MT = Microtubule

SCFA = Short-chain Fatty Acids

b-SCFA= Branched Short-chain Fatty Acids

o-SCFA = Other Short-chain Fatty Acids

Spp. = *Species pluralis*

TI = Tail Intensity

1. Introduction

Human intestinal microbiota is a complex community of micro-organisms which take part in many important processes in the host. They supply energy from undigested substrates, produce vitamins important for the host, resist the colonization of pathogenic microorganisms, and build the host's immune system. In healthy individuals these commensal microorganisms are well balanced (eubiosis), but this situation can be disrupted by different factors such as antibiotic intake, GI tract infections/diarrhoea, GI surgery or starvation (anorexia) [Van Loo *et al.* 2005]. Also, chronic illnesses, stressful lifestyle, and poor diet can imbalance the immune system [Van Loo *et al.* 2005]. Imbalance (dysbiosis) in microbiota can cause overgrowth of bacteria, increase permeability of the intestinal mucosal barrier and enhance deficiencies in host immune defences [Berg 1999]. Cancer development is closely tied to host inflammation which also leads to increased vulnerability to pathogens. Dysbiosis has been shown to contribute to pathogenesis of colon, gastric, oesophageal, pancreatic, laryngeal, breast, and gallbladder carcinomas [Sheflin *et al.* 2014].

The bacterial population in the human intestinal microbiota varies depending on the location and can reach up to 10^{12}-10^{14} per gram of intestinal contents in the colon [von Wright and Salminen 1999]. These communities are especially diverse in terms of community membership [Human Microbiome Project Consortium 2012]. From over fifty phyla represented, only a few are dominant. Among these are Bacteroidetes, Actinobacteria, and Firmicutes [Eckburg *et al.* 2005]. The dominant genera are *Bacteroides, Bifidobacterium, Clostridum,* and *Eubacterium*. However, *Lactobacillus, Enterococcus* and *Enterobactericeae* are present in large numbers [von Wright and Salminen 1999]. The intestinal bacteria have several metabolic functions such as formation of various fermentation end-products, modification of bile acids and metabolic transformation of xenobiotic compounds. They also form a protective barrier against intestinal pathogens [von Wright and Salminen 1999]. Over the past three decades several studies have been addressing the issue as to whether the lactic acid bacteria and/or bifidobacteria can promote human health. Many of these bacterial strains are called **probiotics**.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide. In 2012 there were 694 000 CRC deaths reported [Steward and Wild 2014]. Colorectal cancer (CRC) is developed through a long multi-stage process, starting from precancerous tumours (polyps), with 5% of the cases evolving into malignant tumours [Pearson *et al.* 2009].Risk factors for CRC are both environmental and genetic. The intestinal environment is considered to play an important role both in colorectal tumour development and the evolution and modulation of mucosal immunity. Initiation of CRC could be through production of carcinogens, cocarcinogens, and procarcinogens. Diet and lifestyle factors such as

smoking, stress, obesity, and physical inactivity play an important role. There is an interaction between dietary factors, colonic epithelium, and intestinal microbiota [Adolfsson *et al.* 2004]. Diets high in fat and red meat consumption and low in vegetables increase the faecal excretion of nitroso compounds (genotoxic substances) [Hughes *et al.* 2001] and the genotoxic potential of faecal water (FW) [Rieger *et al.* 1999]. The intestinal environment is exposed in potentially procarcinogenic and carcinogenic substances derived from the dietary or endogenous residues, after being fermented by the gut microbiota. The release of these substances in human faeces and their transformation into genotoxic and mutagenic material has facilitated the study of intestinal exposure on the diet and gut microbiota components [Gratz *et al.* 2011]. Furthermore, CRC development is associated with oxidative stress. The assessment of oxidative stress and the administration of antioxidant**s** are importantfor the treatment and prevention of colorectal cancer [Chang *et al.* 2008].

In this complex ecosystem some overgrown bacterial strains, isolated or acting concurrently, are considered to possibly be playing a negative role in the health of the host. Evidence from several studies suggested this may happen by pro-inflammatory and metabolic stimulus. Microbiota could promote CRC development by inducing a chronic inflammatory state, through the biosynthesis of genotoxins interfering with the cell cycle regulation or directly damaging DNA, through the production of toxic metabolites, and by converting dietary heterocyclic amines to pro-carcinogenic compounds [Candela *et al.* 2014]. Enterobacteria, bacteroides and clostridium can act as opportunistic pathogens, and they have been observed to convert pro-carcinogen compounds into carcinogens by producing β-glucuronidase and β-glucosidase enzymes [Nakamura *et al.* 2002].

Interventional studies are often designed to increase the population of the so-called beneficial bacteria, such as bifidobacteria and lactobacilli. One way to push the balance towards richer composition of beneficial bacteria is by consuming **prebiotics**. Prebiotics are non-digestible food components which have a beneficial effect on the host by selectively stimulating the growth and/or activity of bacterial strains already resident in the intestinal cavity [Gibson and Roberfroid 1995]. Prebiotics are not digested by the gastrointestinal enzymes. Instead, they pass through the upper intestinal tract into the intestinal lumen where they are selectively fermented by the beneficial bacteria [Gibson and Roberfroid 1995].

Several new types of oligosaccharides have been discovered and some of these are considered to have beneficial health effects. β-glucans are polysaccharides occurring in the bran of cereal grains, mainly in barley and oat, and also in different types of mushrooms, seaweed and fungi [Wood 2007]. They are mainly linear β-(1/3)/(1/4)-D-glucopyranosyl polymers referred to as mixed-linked or cereal β-glucans [Westerlund *et al.* 1993]. Barley β-glucan is safe [Jonker *et*

al. 2009], well tolerated [Mitsou *et al.* 2010, Turunen *et al.* 2011], and practically completely fermented selectively by the caecum and colon microbiota [Topping and Clifton 2001]. Major fermentation products are short-chain fatty acids (SCFA) [Daniel *et al.* 1997, Wetvicka *et al.* 1996], such as butyrate which is thought to have a role in CRC prevention [Zhang *et al.* 2010, Roy *et al.* 2009, Kopp *et al.* 2009]. Cereal β-glucans exert immune-modulating activity *in vitro* [Wetvicka *et al.* 1996, Cheung *et al.* 2002, Ramakers *et al.* 2007, Tada *et al.* 2009] and *in vivo* [Yun *et al.* 2003], both on leukocytes and enterocytes. Also, there is evidence concerning their antitumor activity from animal studies, but the data from human studies is limited [Cheung *et al.* 2002, Cheung and Modak 2002].

The inverse association between pro- and prebiotics and CRC is derived from *in vitro*, animal, epidemiological, and human interventional studies, evidence being mainly based on animal models. There is only limited data provided by interventional trials with humans, which unlike the epidemiological studies provide information on the causal relationship between nutritional factors and colon cancer. The role of pro- and prebiotics in the reduction of CRC in humans is not yet established due to the impractical end-point of cancer in terms of number of subjects, cost, study duration (long lag-phase between exposure to carcinogenic stimuli and appearance of tumours), and ethical considerations [Rowland 2004]. In animal models the colorectal cancer treatment reduces the prevalence of the disease (such as tumor incidence, multiplicity and volume, cell proliferation, duration of latency period, survival, formation of aberrant crypt foci (precursor lesion of CRC), faecal bacterial enzyme activity, and antigenotoxicity against N-nitro-N-nitrosoguanidine-, 1,2-di-methylhydrazine- and azoxy-methane-induced genotoxicity) [Van Loo *et al.* 2005, Roller *et al.* 2004, Klinder *et al.* 2004, Nakanishi *et al.* 2003], when in the human models the treatment is shown to reduce the amount of genotoxic substances in faeces [De Preter *et al.* 2008, Sairanen *et al.* 2007, Bouhnik *et al.* 1996a, Bouhnik *et al.* 1996b].

The relationship among microbes, and between the microbiome and clinical parameters [Eckburg *et al.* 2005], and the mechanisms by which pro- and prebiotics affect CRC carcinogenesis are still unclear. The mechanisms that may be involved include enhancement of the host's gut immune response, suppression of harmful intestinal bacteria, sequestration of potential mutagens, production of anti-mutagenic compounds, reduction of pH concentrations in the colon, and alterations of other physiological conditions (such as increase in stool water content, volume, or frequency) [Ishikawa *et al.* 2005, Olivares *et al.* 2006].

To our knowledge, no clinical studies have been conducted on humans that investigated the effect of cereal β-glucan on the gut microbiota of polypectomised patients or CRC patients. Cereal β-glucans exhibit among many other health benefits protective effects against colon

carcinogenesis. Polypectomised patients have an increased CRC risk, possible due to intestinal dysbiosis. It is important to study their microbiota and to examine whetherbarley β-glucan intervention can alter or attenuate this dysbiosis.

2. Colorectal cancer (CRC)

Colon cancer, colorectal cancer, and rectal cancer are all the same disease. The colon and the rectum together form the lower part of the intestinal tract. Colorectal cancer (CRC) is one of the leading causes of death in USA and Western Europe, being the third most common cancer type [Steward and Wild 2014]. Over 90% of the CRC cases are sporadic in nature and the most common type (95%) is adenocarcinoma, which develops from glandular cells lining the wall of the bowel. The majority of sporadic CRC occur in the left side of the colon [Rabeneck *et al.* 2003]. Approximately 3-5% of CRC are associated with high-risk, inherited colon cancer syndromes such as Adenomous and Hamartomatous Polyposis syndromes, MUTYH-associated Polyposis, and Hereditary Nonpolyposis and Common Familial Colon Cancer [Kaz and Brentnall 2006]. Lifestyle factors, such as the combination of dietary factors (fibre, fish, red, and processed meats) in addition to alcohol intake, obesity, and low physical activity play a major role in CRC aetiology [World Cancer Research Fund/American Institute for Cancer Research 2007]. The role of the microbiome in CRC development is under investigation on-going research and the latest data is suggesting that the gut microbiome might be the next candidate biomarker for early detection of colorectal cancer [Zackular *et al.* 2014].

2.1 Etiology

CRC begins with a single cell that changes forms and grows into a visible polyp (a mass of cells projecting from the colon wall). Localized precancerous adenomatous polyps (ademonas) develop in the colon and progress into invasive and metastatic cancerous tumours (carcinomas) over time (Fig. 1). A tumor of the colon is usually first detected as a polyp or as smaller precursor lesions of CRC, aberrant crypt foci [Hope *et al.* 2005].The mechanisms of how a polyp can progress into cancer are unknown. It takes approximately 2 to 7 years for a polyp to become cancerous. Thus, early detection at the adenoma stage of this disease has been critical for successful treatment and survival. If polyps are found during the early stage of becoming cancerous, surgical procedure (polypectomy) can be done to remove them and to prevent further spreading. Early detection and removal of adenomas can prevent CRC with a survival rate exceeding 90%. However, there is a dramatic decline in survival following invasion and metastasis [Surveillance, Epidemiology, and End Results (SEER) Program Research Data 2013].

Usually CRC stays asymptomatic until it has advanced to the stage of cancer. Some common symptoms that a CRC patient may experience are bleeding, pain, unexpected weight loss, and a change in bowel habits.

Some polyps progress to cancer whilst other do not, indicating that there are several factors influencing the onset of the disease. Such factors can be environmental, diet, lifestyle (smoking, physical activity level, stress, obesity), genetic susceptibility (inheritance), colonic microbiota (high level vs. low level of pathogenic or probiotic bacteria), and aetiological [inflammatory bowel disease, papillomavirus and acquired immunodeficiency syndrome (AIDS)].

According to Borges-Canha *et al.* two major pathways for colorectal carcinogenesis are now suggested: the APC/β-catenin pathway (chromosomal instability) and the microsatellite instability pathway (associated with DNA mismatch repair genes) [Borges-Canha *et al.* 2015]. The first more common pathway, appears as a consequence of mutations on oncogenes and tumor suppressor genes, and represents about 85% of colonic sporadic tumours. In the second pathway (about 15% of sporadic colonic tumours) the mismatch repair genes (mostly MLH1 and MSH2) are inactivated and a precursor lesion might not be apparent [Cunningham *et al.* 2010, Poulogiannis *et al.* 2010, Peltomäki 2003].

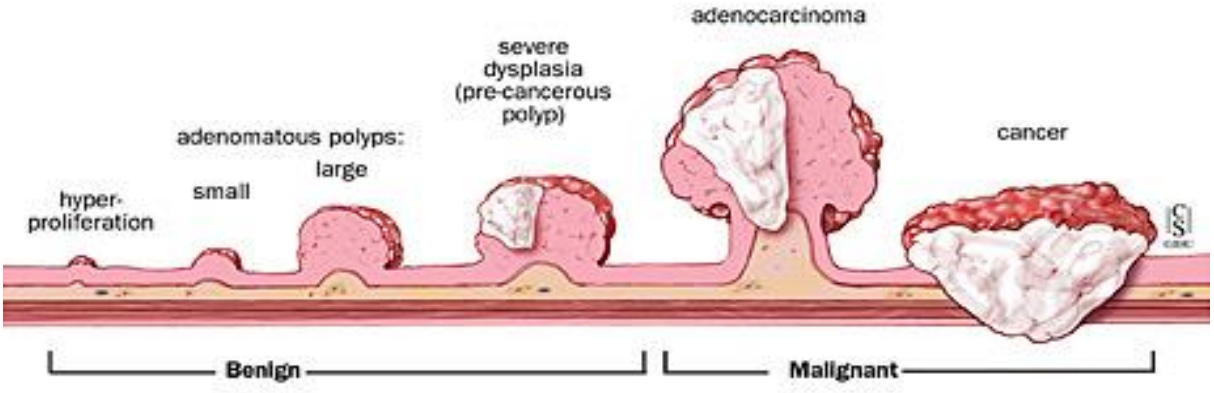


Figure 1.CRC development. CRC begins with a single cell that changes forms and grows into a visible polyp. Localized precancerous adenomatous polypsdevelop in the colon and progress into invasive and metastatic cancerous tumours overtime.

2.2 Colorectal cancer and diet

Dietary factors can be both protective and harmful. Meta-analysis from epidemiological studies have provided a list of nutrients identified as chemopreventive agents in CRC, such as a wide group of polyphenols (i.e. green tea polyphenols, soy flavonoid genistein, quercetin from onions, resveratrol from grapes or curcumin), garlic (organo-sulfur compounds, flavonoids, selenium), polyunsaturated fatty acids (n-3 PUFAs), carotenoids (i.e. β-carotene, lycopene or lutein), vitamins (B6) and minerals (Mg) [Hou *et al.* 2013, Langner and Rzeski 2012, Magalhães *et*

*al.*2012]. There is less consistent evidence for fruit and vegetable intake (fibre and folate), fish and Omega-3 fatty acids, selenium, dairy, calcium, and vitamin D [Hou *et al.* 2013]. Also, active living, maintaining a healthy weight and waist circumference significantly protect against developing colorectal cancer [Hou *et al.* 2013].Diets high in meat (especially beef and fried meet), fat (n-6 polyunsaturated fatty acids (PUFA)), iron, and alcohol increase the risk of CRC. Smoking is also a risk factor of CRC occurrence[Zackular *et al.* 2014, Hou *et al*. 2013, Magalhães *et al.* 2012, Hughes*et al*. 2001].One theory is that risks attributable to foods are mediated by bacterial actions in the intestine. A meal high in fat intake increases the bile acid production. Bacteria are known to reduce bile acids, deconjugating 7 α-hydroxyl groups to produce cytotoxic 7 α-dehydroxylating bile acids deoxycholate and lithocholate. These compounds are shown to promote cell proliferation and growth of adenomas [Nagengast *et al.* 1995, Govers *et al.* 1996], to induce cell necrosis, hyperplasia, and to alter DNA synthesis in the intestinal mucosal cells [Gill and Rowland 2002]. Numerous human studies report a correlation between faecal bile acid and CRC [Azcárate-Peril *et al.* 2011, Imray *et al.* 1992, de Kok *et al.* 1999]. High meat intake increases the amount of the toxic substance ammonia in the faeces which is produced by the intestinal bacteria after catabolizing protein and urea. A low level of ammonia in the gut is associated with low protein and high-fibre diets, which appear to protect against CRC [Gill and Rowland 2002, Shen *et al*. 2012]. Carcinogenic heterocyclic amines (HCA) are formed during the cooking of protein-rich foods. They are activated into mutagens and carcinogens, such as the HCA 2-amino- 3-methylimidazo[4,5-f]quinoline (IQ) in the liver [Nagao and Sugimora 1993]. In the colon *Eubacterium* and *Clostridium* genera activate HCA IQs into its derivate 7-hydroxy-IQ, which is genotoxic to the colon cells [Van Tassel *et al.* 1990].

The activity of faecal carcinogenic bacterial enzymes such as β-glucuronidase, nitroreductase and azoreductase can also be altered by diet**.** For example a high fibre intake (wheat bran, oat bran, whole-meal rye) has been demonstrated to reduce bacterial β-glucuronidase [Reddy *et al.* 1999, Shen *et al*. 2012], azoreductase [Shen *et al.* 2012] and β-glucosidase activity [Gråsten *et al.* 2000] both in animals and humans. Compared to vegetarians, omnivores eating western type of diet have higher levels of β-glucuronidase, nitroreductase, and azoreductase [Goldin *et al*. 1980].

The inverse association between probiotics, prebiotics, synbiotics and CRC is derived from *in vitro*, animal, epidemiological, and human intervention studies. However, the evidence is mainly based on animal models.The data provided by interventional trials with humans is limited, and unlike the epidemiological studies, suggests a causal relationship between nutritional factors and colon cancer. In animal models the colorectal cancer treatment by pro- and prebiotics reduces the prevalence of the disease (such as tumor incidence, multiplicity and

volume, cell proliferation, duration of latency period, survival, formation of aberrant crypt foci (precursor lesion of CRC), bacterial enzyme activity from the faeces, and antigenotoxicity against N-nitro-N-nitrosoguanidine-, 1,2-di-methylhydrazine- and azoxymethane-induced genotoxicity) [Van Loo *et al.* 2005, Roller *et al.*2004, Klinder *et al.* 2004, Nakanishi *et al.* 2003]. In humans the treatment is shown to reduce the amount of genotoxic substances in faeces [De Preter *et al.* 2008, Sairanen *et al.* 2007, Bouhnik*et al.*1996a, Bouhnik *et al.* 1996b].

The mechanisms by which probiotics, prebiotics, and synbiotics affect CRC carcinogenesis are still unclear. They have the potential to inhibit the development and progression of neoplasia by decreasing intestinal inflammation while enhancing the host's gut immune response and antitumorigenic activity.They are able to bind to potential food carcinogens including toxins found in meat products, reduce bacterial enzymes which hydrolyse precarcinogenic compounds such as β-glucuronidase, suppress harmful intestinal bacteria, produce anti-mutagenic compounds, reduce colonic pH, and alter other physiological conditions (such as increase in stool water content, volume, or frequency) [Geier *et al.* 2006, Ishikawa *et al.* 2005, Olivares *et al.* 2006].

The human interventional studies on colon cancer are usually separated into studies on lactic acid producing bacteria's (LAB) ability to bind food mutagens, and/or their ability to alter the activity of enzymes related to colon carcinogenesis. These studies usually use as biomarkers the activities of the following carcinogens releasing bacterial enzymes; β-glucuronidase [de Preter *et al.* 2008, Ling *et al.* 1994], β-glucosidase [Wollowski *et al.* 2001, de Preter *et al.* 2008], nitroreductase [Ling *et al.* 1994, Golding and Gorbach 1984], and azoreductase[Golding and Gorbach 1984, Pedrosa *et al.* 1995]. Also, an alteration of physico-chemical conditions in the colon, such as pH [Lidbeck *et al.* 1992, de Kok *et al.* 1999], soluble bile acid concentration [de Kok *et al.* 1999, Hague *et al.* 1995], sort-chain fatty acid concentration [Hague *et al.* 1995], and mutagenic activity of faecal water have been under the investigation [de Kok *et al.* 1999, Oberreuther-Moschner *et al.* 2004, Oβwald *et al.* 2000]. A few interventional studies have also been carried out using recurrence of colonic adenomas as an end-point and colonoscopy as a tool [Rafter *et al*. 2007, Gill and Rowland 2002]. Recent research focuses on the structural changes of CRC patients gut microbiota compared to healthy individuals and on the interplay between endogenous and environmental factors, such as diet [Candela *et al.* 2014, Zackular *et al.* 2014, Viljoen *et al.* 2015, Azcárate-Peril *et al.* 2011].

3. Gut microbiota

Outnumbering human cells 10 to 1, over 100 trillion microbes are hosted in the human body, the majority of them inhabiting the gut in a continuum of dynamic ecological communitiesreferred

to as microbiome [Candela *et al.*2014]. The intestinal habitat consists mainly of bacteria (~10^{14}) and contains more than 10^6 bacterial genes [Human Microbiome Project Consortium 2012]. The intestinal colonisation occurs immediately after birth with strong influence from the maternal gut microbiota [Thomas *et al.* 2014].The stomach and small intestine contain only a few species of bacteria adhering to the epithelia and some other bacteria in transit [Guarner and Malagelada 2003]. In contrast, the large intestine is colonized by a dense and complex community composed largely of anaerobic bacteria outnumbering the aerobic bacteria. Their cell number can exceed 10^{11} per gram [Flint *et al.* 2007]. Microbiome diversity remains unexplained although diet, environment, host genetics, age, and early microbial exposure have all been implicated [Human Microbiome Project Consortium 2012]. Several studies suggest that a gut microbiota with low diversity may have negative consequences for health [Thomas *et al.* 2014]. Recent development in metagenomics has rendered several international human microbiome projects possible (the MetaHIT project in the European Union and China, the MicroObes project in France, the Meta-GUT project in China) in order to enlist microbial species and better understand their role in cancer development.

The anaerobic genera of *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus* are known to be predominant in humans, whereas aerobic genera such as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsielle*, *Lactobacillus*, *Proteus* etc. are subdominant [Guarner and Malagelada 2003]. According to the latest studies using metagenomic techniques the dominant divisions are Cytophaga-Flavobacterium-Bacteroides (Bacteroidetes). *Bacteroides* and Firmicutes (which are mainly Gram positive bacteria, such as the genera *Clostridium* and *Eubacterium*) are the most well-known genera in this phylum and each comprise ~30% of bacteria in faeces and in the mucus overlying the intestinal epithelium [Seksik *et al.* 2003]. The Human Microbiome Project has analysed the largest cohort and set of distinct, clinically relevant body habitats to date. Gut microbiota is characterised by great diversity. For example *Bacteroides* ranged from dominant in some subjects to a minority in others who carried a greater diversity of Firmicutes. *E. coli* was present at >0.1% abundance in 15% of stool microbiomes (>0% abundance in 61%) [Human Microbiome Project Consortium 2012]. Gut bacteria have a major impact upon nutrition and health via the supply of nutrients, conversion of metabolites, and interaction with host cells [Flint *et al.* 2007]. Some of the colonizing bacteria are potential pathogens and can be a source of infections and sepsis when the integrity of the bowel barrier is physically or functionally interrupted.

The large intestine has three parts: cecum (the vermiform appendix is attached to the cecum), colon (ascending colon, transverse colon, descending colon and sigmoid flexure) and

rectum(Fig. 2). The fermentation of non-digestible dietary residue and endogenous mucus occurs mostly in the cecum and right colon. The fermentation produces SCFA as end-products [Wong *et al.* 2006], lowers colonic pH [Johnson *et al.* 2006] and increases the bacterial growth [Mcfarlane *et al.* 1992].

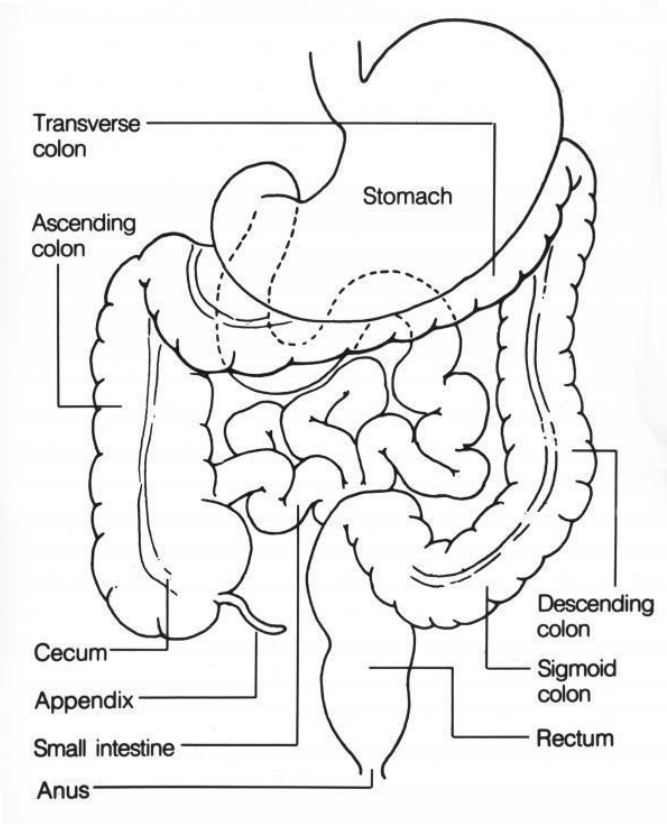


Figure 2.Human gut. The large intestine has three parts: cecum (the vermiform appendix is attached to the cecum), colon (ascending colon, transverse colon, descending colon and sigmoid flexure) and rectum.

Gut microbiota also participates in vitamin synthesis [Hill 1997], and in the absorption of calcium, magnesium, and iron [Younes *et al.* 2001] while controlling epithelial cell proliferation and differentiation (stimulated by SCFA) [Frankel *et al.* 1994]. They also protect against pathogen invasion [Bernet *et al.* 1994].The composition of this microbiome can fluctuate due to acute diarrhoeal illnesses, antibiotic treatment, or by dietary interventions [Simon and Gorbach 1984].

3.1 Gut microbiota, bacterial metabolites and colon cancer

The intestinal environment plays an important role both in colorectal tumor development and in the evolution and modulation of mucosal immunity. The gut microbiome is a collection of symbiotic microorganisms inhabiting the gastrointestinal tract and is associated with diseases

such as obesity and inflammatory bowel disease [Cénit *et al.* 2014, Kinross *et al.* 2011].Based on animal studies, enteric bacteria may contribute to CRC development by interacting through immune system, by producing cancer-associated metabolites, and by releasing genotoxic virulence factors [Zackular *et al.* 2013 and 2014, Arthur *et al.* 2012, Kostic *et al.* 2013] (Fig. 3).

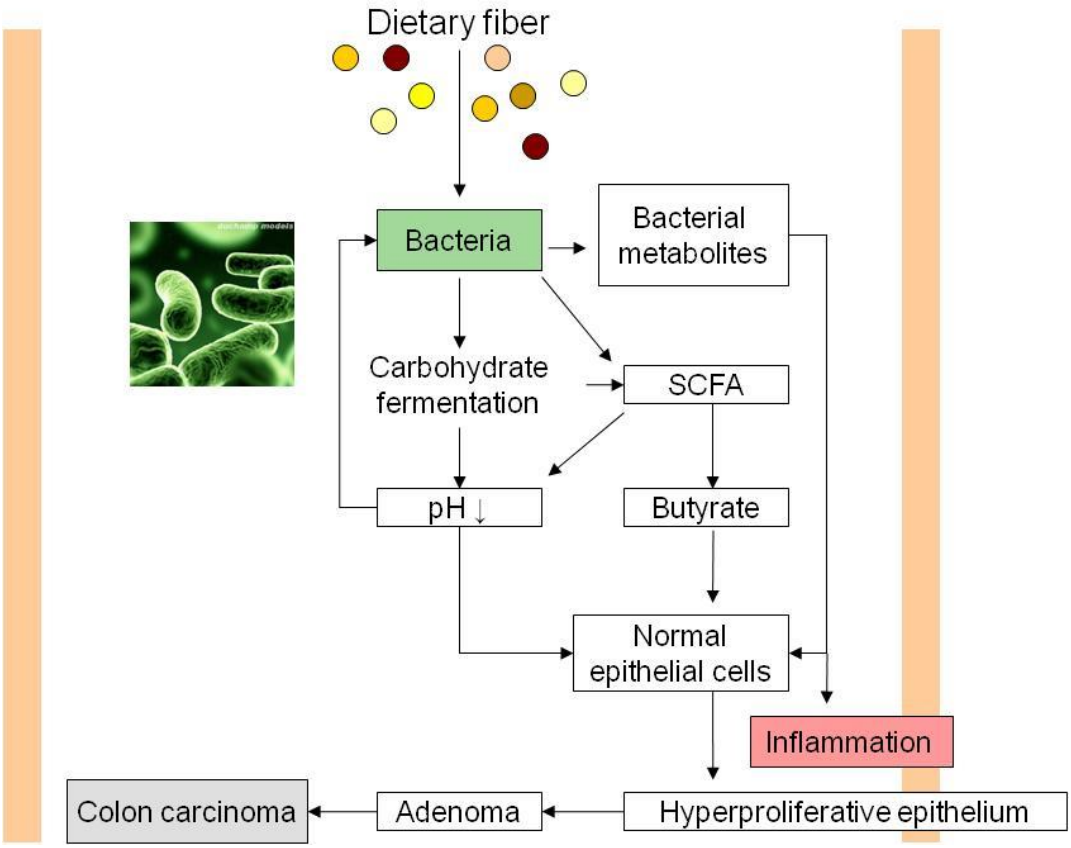


Figure 3.The bacterial metabolism in the colon and CRC development. This image describes the possible effect of dietary fibre on the metabolism of the intestinal bacteria. The carbohydrates are fermented by the intestinal bacteria producing SCFA, such as butyrate, as an end-product. The production rate of SCFA in the colon depends on the quantity and the biodiversity of bacterial strains present, on the fermented substrate and on the transit time. The butyrate is considered to be an important energy source to the colonic cells. Butyrate has many other important and protective functions, such as being a regulator of mucosal gene expression, differentiation and apoptosis. SCFAs act to lower colonic pH, which can inhibit growth of potential pathogens and promote the growth of beneficial bacteria such as bifidobacteria and lactobacilli. The risk of colon cancer is associated with bacterial metabolite (enzymes, toxins) activity of various intestinal bacteria, such as *Bacteroides* spp. and *Clostridium* spp..

Inflammation is a common outcome of various environmental and endogenous triggers and can compromise the microbiota-host mutualism causing an increase in pathobionts at the expense of health-promoting groups. This allows the microbiota to acquire an overall pro-inflammatory configuration [Candela *et al.* 2014]. The involvement of microbiota in the

damaged epithelium has set up an investigational model for inflammation carcinogenesis [Haglund and Soreide 2014].

In CRC development localized precancerous adenomatous polyps (adenomas) displayed in the colon eventually progress into invasive and metastatic cancerous tumours (carcinomas) [Fearon 2011]. Diets high in fat and red meat consumption and poor in vegetables, increase the faecal excretion of nitroso compounds (genotoxic substances) [Hughes *et al.* 2001] and the genotoxic potential of faecal water [Rieger *et al.* 1999] (Fig. 3).

Initiation of colon cancer could be through the production of carcinogens, cocarcinogens, and procarcinogens. There are several biological activities of the intestinal microbiota that can contribute to CRC carcinogenesis by either increasing or decreasing the risk of getting the disease. For example these biological activities can; (1) induce a chronic inflammatory state [through a variety of cytokines and inflammatory mediators, reactive oxygen species (ROS)], (2) increase the biosynthesis of genotoxins interfering with the cell cycle regulation (transformation of secondary bile salt, generation of hydrogen sulphide, β-glucuronidase activity), (3) directly damage DNA through the production of toxic metabolites (production of aglycones, aromatic amines, acetaldehyde, arylsulfatase), and (4) they can convert dietary HCAs to pro-carcinogenic compounds (polycyclic aromatic hydrocarbon-inducible CYPIA2) [Candela *et al.* 2014, Azcárate-Peril *et al.* 2011].

Probiotic activities that may decrease the riskof CRC include; (1) bile salt hydrolase activity and resistance to bile, (2) the generation of microbial metabolites from dietary components (for example equol and/or other beneficial aglycones), (3) reduction of DNA damage (antigenotoxic properties against chemical carcinogens), (4) reduction of the generation of mutagenic or genotoxic compounds (glutathione-S-transferase, glutathione, glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase activity), and (5) the production of bioactive molecules such as short-chain fatty acids (butyrate) [Azcárate-Peril *et al.* 2011].

Data from human studies show that patients with colorectal cancer and polypectomy have an atypical gut microbiome structure and metabolic activity when compared with healthy patients [Zackular *et al.* 2014, Viljoen *et al.* 2015, Gao *et al.* 2015, Kawano *et al.* 2010, Scanlan*et al.* 2008]. The bacterial density varies within the intestine. The large intestine is estimated to contain a 12-fold higher amount of bacteria than the small intestine. Interestingly, there is also a 12-fold higher cancer risk in the large intestine compared to the small intestine [Sobhani *et al.* 2013]. New sequencing technology has made it possible to catalogue microbial species found from different cancer types and sites. For example, differences in microbial and archaeal composition between mucosal samples from healthy and diseased tissue in tubular adenoma and adenocarcinoma were published [Mira-Pasqual *et al.* 2015]. The earlier clinical studies on

intestinal microbiota related to colon cancer did not present consistent results. The most convincing positive correlation frim epidemiological studies was found with the genus *Bacteroides* while the most convincing negative correlation was found with *Lactobacillus*[Moore and Moore 1995, Kawano *et al.* 2010].

In a meta-analysis of 31 original articles, including both human and animal studies, on the role of colon microbiota in CRC development, some bacteria were consistently augmented (such as Fusobacteria, *Alistipes*, *Porphyromonadaceae*, *Coriobacteridae*, *Staphylococcaceae*, *Akkermansia* spp., and *Methanobacteriales*) while others were constantly diminished in CRC (such as *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, *Faecalibacterium* spp., *Roseburia*, and *Treponema*) despite the different methodologies used. The consistent increase of amino acids and decrease of butyrate throughout colonic carcinogenesis were also confirmed [Borges-Canha *et al.* 2015].

In a recent study, the gut microbiome of patients representing the three stages of CRC development (healthy, adenoma, and carcinoma) revealed that there is both an enrichment and depletion of several bacterial populations associated with adenomas and carcinomas. It is proposed that gut microbiota analysis, if combined with known clinical risks factors (age, BMI, race), would improve the ability to detect and differentiate the presence of precancerous and cancerous lesions [Zackular *et al.* 2014].

In another study, the relationship between the presence and the level of colonisation of several bacterial species with site and stage of disease, age, gender, ethnicity and microsatellite instable (MSI)-status was investigated in paired tumour and normal tissue samples from 55 colorectal cancer patients. Enterotoxigenic *Bacteroides fragilis* and afaC-positive *E. coli* were significantly enriched in the colon compared to the rectum and both ETBF and *Fusobacterium* spp. levels were significantly higher in late stage colorectal cancers. No samples were found positive for *S. gallolyticus* [Viljoen *et al.* 2015].

The gut microbiota distribution features of the cancerous and non-cancerous normal tissue in proximal and distal CRC samples have been investigated through a pyrosequencing based analysis of the 16S rRNA gene V3 region [Gao *et al.* 2015]. Firmicutes and Fusobacteria were over-represented whereas Proteobacteria was under-represented in CRC patients. In conclusion, the overall microbial structures of proximal and distal colon cancerous tissues were similar. However, certain potential pro-oncogenic pathogens were different [Gao *et al.* 2015].

Intestinal microorganisms and the possible mechanisms involved in CRC development were extensively reviewed and indicate the possible role of inflammation, bacterial toxins, and toxic microbiota metabolites in CRC onset (Fig. 4) [Candela *et al.* 2014]. Data from over 10 different studies showed that CRC patients, in contrast with healthy controls, were significantly enriched

in faecal *Fusobacterium*, *Enterococcaceae*, *Campylobacter*, *Erysipelotrichaceae*, *Collinsella*, *Peptostreptococcus* and *Anaerotruncus*, and depleted in members of the *Clostridium* cluster IV, such as *Faecalibacterium prausnitzii* and *Roseburia*. On the intestinal mucosa, CRC patients showed an increase of *Porphyromonas*, *Fusobacterium*, *Peptostreptococcus* and *Mogibacterium*, whereas *Faecalibacterium*, *Blautia* and *Bifidobacterium* were depleted. Enriched bacteria in CRC related microbiota, such as *Fusobacterium*, *Enterococcaceae* and *Campylobacter*, can act as pro-inflammatory opportunistic pathogens. Depleted bacteria, such as *F. prausnitzii* and *Roseburia* (well-known butyrate producers) and bifidobacteria (protector) are important microorganisms preserving the intestinal homeostasis [Candela *et al*. 2014] (Table 1).

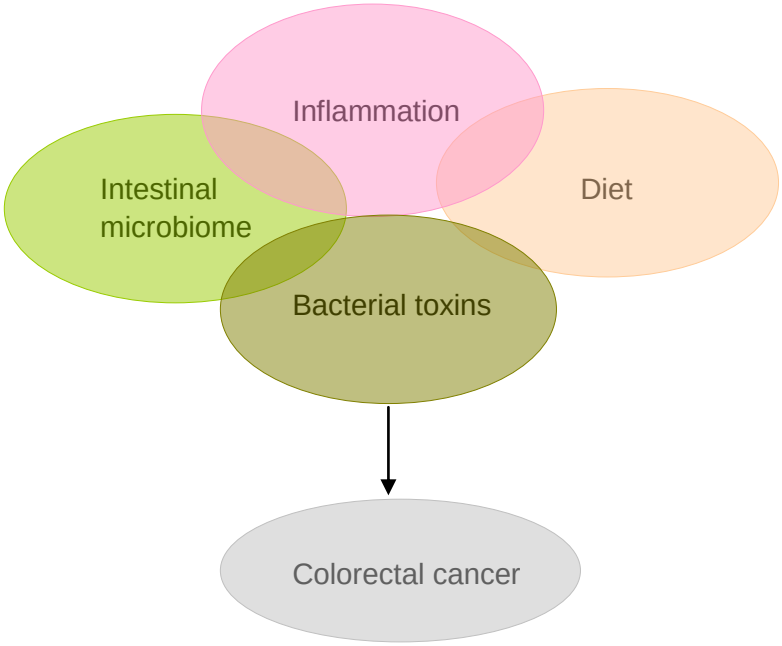


Figure 4.The interplay between endogenous and exogenous factors in CRC development. Colorectal cancer arises from the interplay between endogenous and exogenous factors, such as inflammation, diet, intestinal microbiome structure, and transcription and activity of bacterial genotoxins. Taken from Candela *et al.* Inflammation, gut microbiome and colorectal cancer.Word J Gastroenterol (2014).

Findings from new sequencing technology studies have helped Tjalsma *et al.* to develop the so called driver-passenger model in order to better understand the involvement of intestinal microbiota in the origin and development of CRC. Paying special attention to changes in bacterial composition in microbiota during CRC progression, they proposed that CRC can be initiated by “driver” bacteria which are eventually replaced by “passenger” bacteria that either promote or stall tumorigenesis [Tjalsma *et al.* 2012]. At first, certain bacteria drive the epithelial DNA damage that contributes to the initiation of CRC (termed bacterial drivers). Inflammation in the gut favours the growth of toxigenic bacterial drivers and therefore promotes carcinogenesis [Candela *et al.* 2014].

Table 1. Microorganisms involved in colorectal cancer. Adapted from Candela *et al.*(Inflammation, gut microbiome and colorectal cancer) and updated with the latest data. Word J Gastroenterol (2014).

Microorganism	Role in CRC	Mechanism	Ref.
E. faecalis	Driver	Production of superoxide	Wang *et al.* 2012b, Balamurugan *et al.* 2008, Wang *et al.* 2007, Huycke *et al.* 2002
*E. coli NC*101	Driver	Genotoxin production (colibactin)	Raisch *et al.* 2014, Dalmasso *et al.* 2014, Cougnoux *et al.* 2014, Kohoutova *et al.* 2014 Cuevas-Ramos *et al.* 2010
B. fragilis	Driver	Genotoxin production (fragilisin)	Geis *et al.* 2015, Boleij et al. 2015, Sears *et al.* 2014, Toprak *et al.* 2006
Shigella	Driver	Induction of inflammation	Tjalsma *et al.* 2012, Sperandio *et al.* 2008, Koterski *et al.* 2005
Citrobacter	Driver	Induction of inflammation	Roy *et al.* 2015, Tjalsma *et al.* 2012, Umar 2012
Salmonella	Driver	Induction of inflammation	Pontier-Bres *et al.* 2014, Zhang *et al.* 2013, Tjalsma *et al.* 2012
Enterobacteriaceae	Helper	Induction of inflammation	Murakami *et al.* 2015, Tjalsma *et al.* 2012, Chou *et al.* 2006
Fusobacterium	Passenger	Induction of inflammation	Viljoen *et al.* 2015, Kostic *et al.* 2013 Rubinstein *et al.* 2013
S. gallolyticus	Passenger	Induction of inflammation	Takamura *et al.* 2014 Boleij and Tjalsma 2013
C. septicum	Passenger	Induction of inflammation	Raghavendra e*t al.* 2013 Stecher and Hardt 2008 Wentling*et al.* 2006
F. prausnitzii	Protective	Protective butyrate production; anti-inflammatory properties	Marchesi *et al.* 2011 Balamurugan *et al.* 2008
Roseburia	Protective	Protective butyrate production; anti-inflammatory properties	Zhu *et al.* 2014, Marchesi *et al.* 2011,
Bifidobacterium	Protective	Protections from pathogens; anti-inflammatory properties	Tojo *et al.* 2014 Candela *et al.* 2011
Corynebacteriaceae	Protective	Anti-inflammatory properties	Marchesi*et al.* 2011

Second, tumorigenesis induces intestinal niche alterations that favour the proliferation of opportunistic bacteria (termed bacterial passengers). The bacterial drivers (alpha-bugs and their helpers) are gradually outcompeted by gut commensals with either tumour-promoting or tumour-suppressing properties (bacterial passengers). Suggested candidates for bacterial drivers are intestinal bacteria with pro-carcinogenic features that may initiate CRC development, such as *Enterococcus faecalis*, certain *Escherichia coli* strains, *B. fragilis*, members of the Enterobacteriaceae (*Shigella, Citrobacter* and *Salmonella* spp.) and several enterobacteria.

Suggested candidates for bacterial passengers are gut bacteria such as *Fusobacterium* spp., *Streptococcus gallolyticus* subsp. *gallolyticus*, members of the family *Streptococcaceae*, and *C. septicum* that are relatively poor colonizers of a healthy intestinal tract but have a competitive advantage in the tumour microenvironment, allowing them to outcompete bacterial drivers of CRC. In addition to these opportunistic pathogen passengers, the family *Coriobacteriaceae* (*Slackia* and *Collinsella* spp.), the genus *Roseburia*, and the genus *Faecalibacterium* could act as bacterial passengers of CRC tissue displaying anticarcinogenic activity [Tjalsma *et al.* 2012].

Bacteroides fragilis (driver).There are two classes of *B. fragilis* distinguished by their ability to secrete a zinc-dependent metalloprotease toxin, *B. fragilis* toxin (BFT). Strains that do not secrete BFT are nontoxigenic *B. fragilis* (NTBF), and those that do are called enterotoxigenic *B. fragilis* (ETBF) [Sears *et al.* 2014]. ETBF, fragilysin-producing *B. fragilis*, is a pathogenic variant of a common commensal. They are emerging enteric pathogens associated with diarrhoeal disease and also associated with recurrence of inflammatory bowel disease [Sears *et al.* 2014, Tobrak *et al.* 2006]. ETBF strains have been shown to directly influence the development of colorectal cancer through the production of a metalloprotease toxin [Sears *et al.* 2014], by cleaving E-cadherin bound intracellularly to b-catenins [Hardy *et al.* 2000] and by affecting regulatory T-cells [Geis *et al.* 2015].In a recent study, the presence of the BFT gene in mucosal samples from colorectal neoplasia patients and from healthy controls was compared; the mucosa of colorectal neoplasia cases was significantly more often BFT-positive (89%) compared to healthy control (29%) biopsies [Boleij *et al.* 2015]. Cases of extra- or intra-abdominal infections caused by bacteroides strains, such as peritonitis, bacterial liver abscesses, and pericarditis, have been shown to be associated with CRC and should be considered as sign for a possible silent colonic cancer [Nachimuthu *et al.* 2001, Lonardo *et al.* 1992, Lam *et al.* 1995].

Escherichia coli (driver). A number of *E. coli* strains produce a wide array of toxins, some of which are turning out to be potentially harmful in humans, either directly damaging DNA, specifically disrupting cell signalling, or by modifying the tumor microenvironment [Raisch *et*

al. 2014, Dalmasso *et al.* 2014, Cougnoux *et al.* 2014, Cuevas-Ramos *et al.* 2010, Bonnet *et al.* 2014]. Inflammation promotes the growth of enterotoxigenic *E. coli* (ETEC) strains, increasing their adhesion to the host epithelia [Schwabe and Wang 2012]. According to the data from a recent study analysing mucosal biopsies, the intestinal mucosa of patients with more advanced colorectal neoplasia is colonized with more virulent strains of *E. coli* and higher production of bacteriocins is observed in these patients when compared to those with less advanced colorectal neoplasia [Kohoutova *et al.* 2014]. Earlier studies have shown an unexplained inverse relationship between the incidence of CRC and ETEC infections [Carrithers 2003, Ferlay *et al.* 2001]. Pitari *et al.* introduced an interesting hypothesis that specific peptides elaborated from ETEC infections may prevent the hyperproliferative and neoplastic development of intestinal epithelial cells that are associated with initiation and progression of CRC. They found evidence of the presence of a novel intracellular signalling pathway initiated by these specific peptides that prevents proliferation of CRC cells by inhibiting the DNA synthesis in colon carcinoma cells [Pitari *et al.* 2003].

Enterococcus faecalis (driver). E. faecalis is a pro-inflammatory opportunistic pathogen in human intestinal commensal. It produces extracellular superoxide, which can lead to epithelial cell damage and increased turnover [Balamurugan *et al.* 2008, Huycke *et al.* 2002] and also promote chromosome instability via macrophage-induced bystander effects [Wang *et al.* 2012b, Wang *et al.* 2007]. CRC patients, in contrast with healthy controls, were significantly enriched in *Enterococcaceae*, especially in *E. faecalis* [Balamurugan *et al.* 2008, Wang *et al.* 2007]. In an animal study, Interleukin-10 knockout mice colonized with superoxide-producing *E. faecalis* developed inflammation and colorectal cancer, whereas colonization with a superoxide-deficient strain resulted in inflammation but not cancer [Wang *et al.* 2012b].

Enterobacteriaceae members (*Shigella*, *Citrobacter* and *Salmonella* spp.) (drivers). An unbalanced microbiota favour the growth of pro-inflammatory opportunistic pathogens, such as *Enterobacteriaceae* [Lupp *et al.* 2007] and a higher presence of *Enterobacteriaceae* was found by qPCR in samples from CRC compared to healthy controls[Mira-Pasqual *et al.* 2015]. *Shigella, Citrobacter*, and *Salmonella* can act as bacterial drivers and support carcinogenesis by the induction of a pro-inflammatory response [Candela *et al.* 2014, Tjalsma *et al.* 2012]. They are associated with the early stages of CRC, including adenomas, and then disappear from cancerous tissue as the disease progresses [Tjalsma *et al.* 2012]. These genera were first recognized as aetiological agents of human diarrhoeal disease [DuPond 2009]. *Shigella* spp. is over-represented in the non-malignant mucosa-associated microbiome of patients with adenomas

[Shen *et al.* 2010]. *Citrobacter rodentium* infection promotes crypt hyperplasia and tumorigenesis by aberrantly regulating Wnt/β-catenin signalling [Roy *et al.* 2015, Umar 2012]. Antibody titres against flagellin from *Salmonella* spp. are significantly higher in patients with early-stage CRC than in healthy individuals. This finding could be suggestive of increased gut colonization and subclinical infection in individuals who are susceptible to the development of colonic malignancies [Tjalsma *et al.* 2012**]**. *Salmonella* infection may elevate a tight junction protein claudin-2 levels in intestinal epithelial cells. Claudin-2 is associated with the *Salmonella*-induced elevation of cell permeability and contributes to inflammatory bowel disease and colon cancer development [Zhang *et al.* 2013].

Salmonellatyphimurium has been indicated to be involved in tumour progression also by preventing it (helper bacteria). An oral anti-tumour vaccine made of *Salmonella typhimurium* carrying plasmid DNA encoding tumour-associated antigens has been under preclinical development. This oral DNA vaccine significantly increased the life span of tumour-challenged mice in CRC tumour models [Chou *et al.* 2006]. Soon after a tumour-targeting *S. typhimurium*A1-R strain was developed with an ability to inhibit or eradicate primary and metastatic tumours as monotherapy in nude mouse models. Adjuvant treatment with *S. typhimurium* A1-R strain was highly effective in increasing survival and disease-free survival after bright-light surgery of liver metastasis [Murakami *et al.* 2015].

Fusobacterium nucleatum (passenger). *Fusobacterium* spp. can act as pro-inflammatory opportunistic pathogens (passenger bacteria) in CRC related microbiota and have been reported to be enriched on the surface of tumours compared with adjacent healthy tissue [Gao *et al*. 2015, Mira-Pasqual *et al.* 2015, Kostic *et al.* 2013, Rubinstein *et al.* 2013, McCoy *et al.* 2013, Castellarin *et al.* 2012]. In a recent profiling study of colorectal cancer-associated bacteria, *Fusobacterium* were the most commonly detected bacteria, with levels being significantly higher in late stage colorectal cancers [Viljoen *et al.* 2015]. *Fusobacterium* may contribute to tumour multiplicity through the recruitment of immune cells to tumours [Kostic *et al.* 2013, Rubinstein *et al.* 2013], and it may be a marker for the presence of tumours [Zackular *et al.* 2014].

Streptococcus gallolyticus subsp. *gallolyticus* (formally known as *S. bovis*) (passenger). *Streptococcus gallolyticus* subsp. *gallolyticus* and other members of the family *Streptococcaceae* are opportunistic pathogen passengers related to CRC development and they have been reported to support carcinogenesis by the induction of a pro-inflammatory response [Candela *et al.* 2014, Wentling *et al.* 2006]. It is present approximately in 20-50% of colon tumours and less than 5% in the normal colon [Gao *et al.* 2015]. *S. gallolyticus gallolyticus* exploits its unique range of

virulence features to cause symptomatic infections in patients with CRC dependent on pre-existing colonic abnormalities [Boleij and Tjalsma 2013]. It is a passenger bacterium, clinically linked to human endocarditis and colon cancer [Takamura *et al.* 2014, Boleij and Tjalsma 2013], suggesting that colonoscopies should be routine while scanning for colorectal cancer in all patients with *S. bovis* bacteraemia [Takamura *et al.* 2014, Su *et al.* 2013].

Clostridia (passenger). Earlier studies had given evidence that clostridia, especially *Cl. septicum, Cl. perfringens, and Cl. difficile*, are related to CRC carcinogenesis [Raghavendra *et al.* 2013, Stecher and Hardt 2008, Wentling *et al.* 2006, Sungkanuparph *et al.* 2002, Nakamura *et al.* 2002, Rechner *et al.* 2001, Bodey *et al.* 1991]. They possibly act as opportunistic pathogens (passenger bacteria) and may be involved in tumour progression through the induction of a pro-inflammatory response [Stecher and Hardt 2008, Wentling *et al.* 2006, Candela *et al*. 2014]. Several cases have been published of infections caused by clostridia, leading to the diagnosis of adenocarcinoma of the colon suggesting an association of bacteraemia and colon carcinoma [Sungkanuparph *et al.* 2002, Rechner *et al.* 2001]. While examining the intestinal microbiota in patients with colorectal cancer and with polypectomy, a significant increase in populations of *Cl. leptum* and *Cl. coccoides* was discovered in both groups compared to controls [Scanlan *et al.* 2008].

Lactobacillus spp. (passenger). Both animal and human studies have shown a decrease in *Lactobacillus* strains in faecal samples of tumour patients compared to healthy controls [Zhu *et al.* 2014, Mira-Pascual *et al.* 2015]. Also epidemiological studies on the prevention of colorectal tumours have reported convincing negative correlation with genus *Lactobacillus* and CRC [Kawano *et al.* 2010]. The ability of *L. casei* to prevent CRC was investigated on 398 subjects with a history of colorectal tumours. After 4 years of administration, the incidence rate of tumours with a grade of moderate atypia or higher was significantly lower [Ishikawa *et al.* 2005].

Among other bacteria tested, lactobacilli levels were found to be significantly higher in healthy compared to CRC rats [Zhu *et al.* 2014]. In another animal study lactic acid producing bacteria L. *gasseri*, L. *acidophilus*, L. *confusus*, S. *thermophilus*, B. *breve* and B. *longum* were investigated for their ability to inhibit the induction of DNA damage by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or 1,2-dimethylhydrazine (DMH). All strains were antigenotoxic toward MNNG after a single dose of 10^{10} viable cells/kg body weight p.o. 8 hrs before the carcinogen. L. *gasseri* is also shown to decrease the activity of faecal bacterial enzymes β-glucuronidase, nitroreductase, and azoreductase, which are believed to take part in the colon carcinogenesis [Pedrosa *et al.* 1995].

Bifidobacterium spp. (passenger). Several health-promoting effects have been attributed to some specific strains of this genus. They are considered to be protectors against pathogens involved in CRC development [Fanning *et al.* 2012] and to possess anti-inflammatory properties [Candela *et al.* 2011 and 2014]. The data from sequencing studies has shown depleted number of bifidobacteria in CRC-patients compared to controls [Mira-Pascual *et al.* 2015]. In another study the analysis of colorectal tumour samples revealed that along with the enrichment seen in typical driver-passenger bacteria, an enrichmentwithpossible protectors,such as *Bifidobacterium longum* and *Clostridium butyricum* also occurred [Terzić *et al.* 2010].The role of bifidobacteria in CRC prevention was shown by earlier studies as well [You *et al.* 2004, Kim *et al.* 2003, Pinto *et al.* 1983]. B. *longum* has also been widely studied for its role in CRC prevention. In animal models B.*longum* administration was shown to increase the survival time [Lee *et al.* 2004], prevent DNA induced damage by carcinogens [Pool-Zobel *et al.* 1996], play a role in the reduction of aberrant crypts foci (ACF) occurrence [Rowland *et al.* 1998, Challa *et al.* 1997, Singh *et al.* 1997], and inhibit tumour growth and multiplicity [Singh *et al.* 1997, Reddy 1999, Reddy and Rivenson 1993].

Members of *Coriobacteridae*, *Roseburia*, *Fusobacterium* and *Faecalibacterium*. In the course of the examination the biopsy samples of six patients who underwent resections for primary colon adenocarcinoma, no consistent over-representation of potential pathogenic bacteria in CRC tissue was found. Instead, species from Coriobacteridae, *Roseburia*, *Fusobacterium* and *Faecalibacterium*, generally regarded as gut commensals, with probiotic features were observed [Marchesi *et al.* 2011]. *F. prausnitzii*, *Roseburia and Corynebacteriaceae* are known butyrate-producers; therefore, their over-representation may be due to dramatic physiological and metabolic alterations resulting from colon carcinogenesis itself, which supports the theory that these species may be regarded as CRC passengers [Marchesi *et al.* 2011].Albeit, results from many studies show that *Faecalibacterium prausnitzii* and *Roseburia* spp. Weredecreasedin animal models as well as in CRC patients [Wu *et al.* 2013, Mira-Pascual *et al.* 2015, Balamurugan *et al.* 2008, Wang *et al.* 2012a, Wu *et al.* 2013, Zhu *et al.* 2014]. Reduced numbers of *Collinsella* spp. and *Roseburia* spp. have previously been found in elderly subjects using non-steroidal anti-inflammatory drugs (NSAID) compared to non-users [Mäkivuokko *et al.* 2009], suggesting that also these bacteria need inflammatory niches to optimally colonize the bowel wall [Marchesi *et al.* 2011].

Other intestinal bacteria. CRC patients, in contrast with healthy controls, were significantly enriched also in faecal*Campylobacter*, *Erysipelotrichaceae*, *Collinsella*,*Peptostreptococcu*s and *Anaerotruncus* [Candela *et al.* 2014]. Their role in cancer development is yet to be established.

Yeasts. Although *Candida* species are often present in the normal microbiota, alterations in host defences can lead to development of disease. *Candida* infections, ranging from urinary tract infections to bloodstream infections, are common in patients in the intensive care unit [Corpus *et al.* 2004]. In a case study, the liver biopsy sample of a 62-year-old patient who had fever and abdominal pain and a known history of colon cancer and presence of hepatic metastases was found positive for *Klebsiella pneumoniae*, *Candida albicans* and *Mycobacterium tuberculosis*. This finding may indicate a need for searching not only for the usual pathogens, but also for the coexistence of fungi and mycobacteria in cancer patients [Rafailidis *et al.* 2008].

4. Dietary fibres

Dietary fibres are the indigestible portion of food derived from plants. They are naturally present in different food groups, including cereals, vegetables, fruits, and legumes [de Vries *et al.* 2015]. Chemically, dietary fibres consist of non-starchpolysaccharides such as arabinoxylans, cellulose and many other plant components such as resistant dextrins, inulin, lignin, waxes, chitins, pectins, β-glucans and oligosaccharides [US Department of Agriculture, National Agricultural Library and National Academy of Sciences 2005]. Oligosaccharides are carbohydrates consisting of three to ten monosaccharides, while polysaccharides consist of more than ten mono-saccharides.

According to the Institute of Medicine, fibres in the food supply can be either (1) non-digestible carbohydrates and lignin that are intrinsic and intact in plants or (2) functional fibre consisting of isolated, non-digestible carbohydrates that have beneficial physiological effects in humans [Institute of Medicine 2001].

Soluble dietary fibres are readily fermented in the colon into gases and physiologically active by-products while insoluble dietary fibres are metabolically inert and absorb water throughout the digestive system thereby easing defecation [US Department of Agriculture, National Agricultural Library and National Academy of Sciences 2005]. Insoluble fibre acts by changing the nature of the contents of the gastrointestinal tract and by changing the way other nutrients and chemicals are absorbed [Eastwood and Kritchevsky 2005]. Soluble fibre absorbs water to become a gelatinous, viscous substance. Some types of insoluble fibre have bulking action and are not fermented by the intestinal bacteria. Other types of insoluble fibre, such as resistant

starch, are fully fermented [US Department of Agriculture, National Agricultural Library and National Academy of Sciences 2005].

Intact cereal dietary fibres (ICDF) are derived from any part of the cereal plant, including the kernel, hull, or stalk and are minimally processed, although some degree of processing may be required to obtain the fibre rich portion of the kernel (e.g., milling of bran) or to improve food functionality or safety (e.g., pearling, grinding, or bleaching). Fibres are also extracted, isolated, or made by chemical or enzymatic means, such as the synthesis of fibres from endosperm starch or the enzymatic hydrolysis of long chain fibres into oligosaccharides [de Vries *et al.* 2015].The hard outer layer of a cereal grain kernel is called bran. It is a highly concentrated source of dietary fibre: 100g wheat bran contains 43 g of fibre, rice bran contains 21g, and oat bran contains 15g of fibre [US Department of Agriculture, Agricultural Research Service 2011]. The intake of wheat dietary fibre, specifically wheat bran dietary fibre, improves bowel function. A review of 65 interventional studies on healthy individuals showed increased total stool weight, dry stool weight, and stool frequency, and decreased transit time [de Vries *et al.* 2015].

The possible benefits of a high-fibre diet are (1) normalising bowel movements by increasing the weight and size of the stool and by softening it, (2) maintaining bowel health by lowering the risk of developing haemorrhoids and small pouches in the colon, (3) promoting heart health by lowering low-density lipoprotein, by reducing blood pressure and possible inflammation, (4) reducing the risk of developing type 2 diabetes by slowing the absorption of sugar, and (5) supporting the achievement of healthy weight by being more filling [Anderson *et al.* 2009]. Dietary fibres can also balance intestinal pH and stimulate intestinal fermentation and production of SCFA [Johnson *et al.* 2006, Wong *et al.* 2006].

The daily fibre intake recommendation for adults aged 51 or older, is 30g for men and 21g for women [Institute of medicine 2015]. Most commonly consumed foodstuffs are low in dietary fibre. Higher fibre contents are found in foods such as whole grain cereals, legumes, and dried fruits [Slavin 2013].

5. Prebiotics

The definition of prebiotics was revised recently since one of the criteria for prebiotic properties by Gibson and Robertfroid, improvement of health by selective stimulation of the growth and activity of limited number of colonic bacteria, is difficult to verify [Gibson and Robertfroid 1995]. An answer to the question, how many strains of “positive” bacteria are “a limited number” can hardly be given [de Vrese and Schrezenmeir 2008]. According to the latest definition, a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host

well-being and health [Gibson *et al.* 2004, Robertfroid 2007]. Prebiotics must meet the following criteria proven with *in vivo* experiments in animals and mainly in clinical studies. Prebiotics have to:

- be non-digestible (resisting gastric acid, enzymatic digestion and intestinal absorption),
- be fermented by intestinal microbiota, and
- selectively-stimulate the growth and activity of intestinal bacteria [de Vrese and Schrezenmeir 2008].

According to this only carbohydrates like inulin and oligofructose (OF), (trans-) galacto-oligosaccharides (TOS or GOS) or lactulose fully fulfil the prebiotic criteria. Regarding their effect on the gut microbiota, they exert a bifidogenic action [Mitsou *et al.* 2008, Rafter *et al.* 2007] and decrease the growth of putrefactive/pathogenic bacteria [Rafter *et al.* 2007].

Other health effects of prebiotics, such as prevention of diarrhoea or constipation, modulation of the metabolism of the intestinal microbiota, cancer prevention, positive effects on lipid metabolism, stimulation of mineral adsorption and immunomodulatory properties, are indirect, i.e. mediated by the intestinal microbiota, and therefore less-well proven [de Vrese and Schrezenmeir 2008]. The search for other potential prebiotics is ongoing. B-glucan is under examination to be considered as potential prebiotic.

The following mechanisms for cancer prevention have been proposed for the fermentation of prebiotic carbohydrates: (1) production of SCFA (lactic, acetic, propionic, and butyric acid) during fermentation of prebiotic carbohydrates. A more acidic pH and modulation of the intestinal microbiota, especially growth stimulation of carbohydrate-fermenting bacteria, decreased concentration of putrefactive, toxic, mutagenic, or genotoxic substances and bacterial metabolites, as well as secondary bile acids and cancer-promoting enzymes; (2) butyric acid supports the regeneration of the intestinal epithelium; (3) immune modulation [de Vrese and Schrezenmeir 2008].

The majority of prebiotics in the market are derived from non-digestible oligosaccharides. Very few studies have focused on non-digestible long chain complex polysaccharides, such as β-glucans, in relation to their potential as novel prebiotics.

6. B-glucans

B-glucans are polysaccharides occurring in the branof cereal grains (barley and oats), the cell wall of *Saccharomyces cerevisiae* (baker's yeast) and bacteria, certain types of fungi, seaweed, and many kinds of mushrooms (Reishi, Shiitake, Maitake). B-glucans can be in insoluble (Saccharomyces cerevisiae) or in soluble form (cereal brans). Soluble branched β-1,3-D-glucan is well tolerated [Lehne *et al.* 2005] and it is practically completely fermented in the caecum and

colon by the microbiota [Daniel *et al.* 1997, Topping and Clifton 2001]. Major fermentation products are SCFA, which are important for the colonic microbiota [Daniel *et al.* 1997, Topping and Clifton 2001].

The group of β-glucans, especially β-(1,3)-D-glucans, originated from bacterial, fungal and vegetable sources, have been extensively studied during the last decade. Their structures have a common backbone of beta-(1,3) linked glucopyranosyl residues but the polysaccharidic chain can be beta-(1,6) branched with glucose or integrate some beta-(1,4) linked glucopyranosyl residues in the main chain [Laroche and Michaud 2007]. Their physicochemical properties (particularly gelling capacity) has led into their extensive use not only in food but also in medical, pharmaceutical, and cosmetic applications. These polysaccharides are divided into several classes depending on their structural features.

B-(1,3)-D-glucans and β-(1,3)(1,6)-D-glucans are cell wall components of fungi or excreted as exopolysaccharides by bacteria. They have a common structure comprising a main chain of (1,3)-linked β-D-glucopyranosyl units along with randomly dispersed highly branched side chains of β-D-glucopyranosyl units attached by (1,6) linkages. These β-glucans are limited and expensive due to the lack of efficient processes for their extraction and purification [Laroche and Michaud 2007].

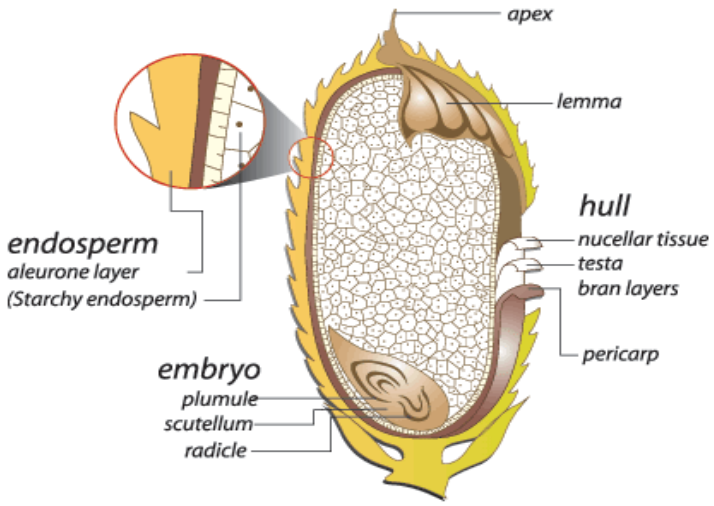


Figure 5. Cereal β-glucansare soluble polysaccharides occurring in the endosperm cell wall of the cereal grains of barley and oats, and to a much lesser degree in rye and wheat.

B-(1,3)(1,4)-D-glucans, cereal β-glucans, are concentrated in cell walls of the starchy endosperm and the aleurone layer of cereals (Fig. 5). They exhibit a different structure having a mixture of beta-(1,3) and beta-(1,4) glucosidic linkages in the main chain [Laroche and Michaud 2007] (Fig.6). These β-glucans can be found in the cell walls of barley [Köksel *et al.* 1999], oats [Wood 1993], wheat [Wood 1997], rye, sorghum [Ramesh and Tharanathan 1998], and many

other cereals. Among all cereal grains, oat and barley contain the highest levels of β-glucans [Charalampopoulos *et al.* 2002] and are presently studied for their high potential in the food industry. The rheological properties of β-glucans appear to depend on a number of factors including the concentration, proportion of branching units, their arrangement [Tosh *et al.* 2004a+b], degree of polymerisation, and their molecular weight [Tosh *et al.* 2004b].

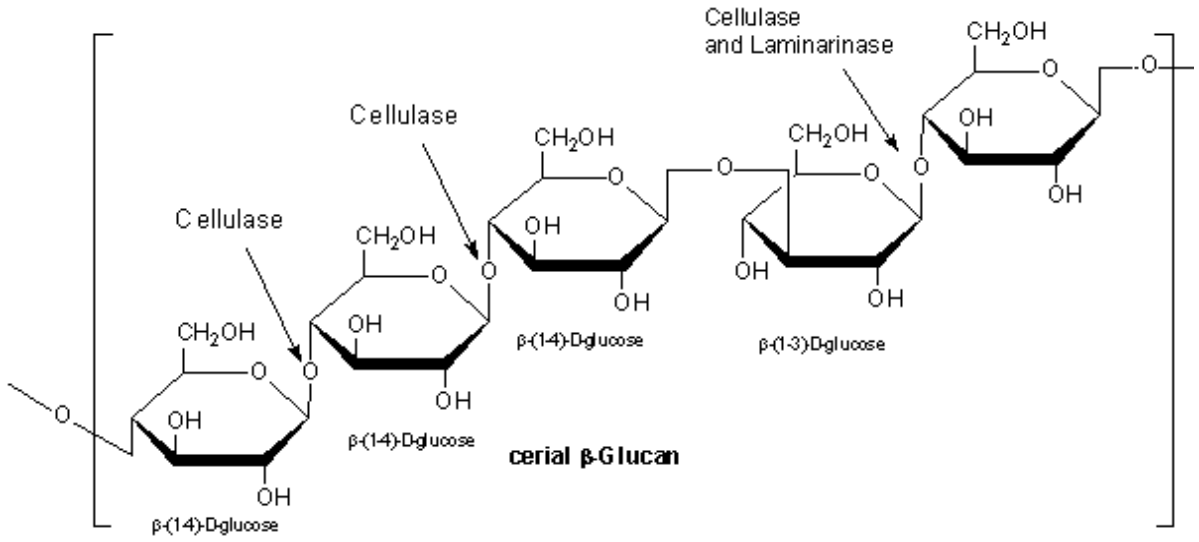


Figure 6. Cereal β-glucans exhibit a different structure having a mixture of beta-(1,3) and beta-(1,4) glucosidic linkages in the main chain.

The non-branched cereal β-glucanis believed to have greater antitumor activity [Mantovani *et al.* 2007, Kiho *et al.* 1998]. According to data from several *in vitro*, animal and human studies, cereal β-glucans can prevent and treat cancer by promoting the growth of beneficial gut microbiota [Mitsou *et al.* 2010, Shen *et al.* 2012, Vasiljevic *et al.* 2007, Snart *et al.* 2006, Drzikova *et al.* 2005, Su *et al.* 2007] thus increasing butyrate concentration [Queenan *et al.* 2007, Nilsson *et al.* 2008] and alleviating constipation commonly caused by CRC therapy [Malkki and Virtanen 2001]. They can, in addition, prevent cancer by acting through immunomodulation (stimulating different immune cells) [Volman *et al.* 2010, Tada *et al.* 2009, Ramakers *et al.* 2007, Cheung and Modak 2002, Větvička *et al.* 1996], by suppressing tumour growth [Akramienė *et al.* 2009], by possessing antimutagenic activity [Angeli *et al.* 2009, Oliveira *et al.* 2006, Cheung *et al.* 2002] and possibly by working as an antioxidant (trapping free radicals) [Agostini *et al.* 2014, Kofuji *et al.* 2012].

There are numerous other health benefits from the ingestion of cereal β-glucans. They can (1) lower LDL and total cholesterol levels [Drzikowa *et al.* 2005, Queenan *et al.* 2007], (2) regulate blood glucose levels (by preventing hyperglycaemia and hyperinsulinemia)

[Thondre and Henry *et al.* 2009, Kim *et al.* 2009, Beck *et al.* 2009, Tapola *et al.* 2005, Jenkins *et al.* 2002], (3) reduce risk factors for obesity [Beck *et al.* 2009] (4) reduce risk factors for cardiovascular diseases (by preventing hyperlipidaemia and hypercholesterolemia) [Talati *et al.* 2009, Keenan *et al.* 2007, Queenan *et al.* 2007, Casiraghi *et al.* 2006, Drzikowa *et al.* 2005], (5) prevent infections and (6) activate phagocytes (by destroying and trapping pathogenic bacteria, viruses, fungi and parasites) [Vizhi and Many 2014, Shin *et al.* 2005].

The prebiotics should be non-digestible, resisting gastric acid, enzymatic digestion and intestinal absorption, fermented by intestinal microbiota, and selectively stimulate the growth and activity of intestinal bacteria [de Vrese and Schrezenmeir 2008]. Since β-glucans are polysaccharides practically completely fermented in the caecum and colon by the microbiota [Daniel *et al.* 1997, Topping and Clifton 2001] and do increase the amount of probiotic bacteria (bifidobacteria and lactobacilli) in the microbiota [Mitsou *et al.* 2010, Snart *et al.* 2006, Drzikova *et al.* 2005, Su *et al.* 2007], theycould be considered prebiotics as well.

Recent studies indicate that prebiotics offer protection against colorectal cancer initiation, mainly due to the elevated SCFA production and more particularly to the production of butyric acid, by the intestinal microbiota. The SCFAs are the main fermentation products of β-glucans by colonic bacteria [Daniel *et al.* 1997, Topping and Clifton 2001]. In a study conducted among healthy volunteers, 8 to 12-weeks of β-glucan consumption increased the butyrate concentration in faeces [Nilsson *et al.* 2008]. Butyrate is considered to be the most important SCFA in colonocyte metabolism, and plays an important role in CRC prevention possibly due to its ability to support the regeneration of the intestinal epithelium [Thomas *et al.* 2014, Zhang *et al.* 2010, Roy *et al.* 2009, Kopp *et al.* 2009, Hinnebusch*et al.* 2002].

Cereal β-glucans are well tolerated. EFSA Panel on Dietetic Products, Nutrition and Allergies has recommended that "in order to bear the claim, foods should provide at least 3g/d of β-glucans from oat, oat bran, barley, barley bran or from mixtures of non-processed or minimally processed β-glucans in one or more servings" [EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 2009]. B-glucans have been incorporated into various products such as bread, muffins, pasta, noodles, salad dressings, beverages, soups, and reduced fat dairy and meat products. Among the cereals cultivated in Europe, wheat is the largest crop, followed by barley and oats in the second and third place [EUROSTAT 2014]. Barley and other cereals are rich sources of fibre, inexpensive, easy to prepare and highly nutritious [Bansal *et al.* 1977].

6.1 Cereal β-glucans and their effect on intestinal microbiota

Data from several studies show that both barley and oat β-glucan selectively support the growth of probiotics Lactobacilli and Bifidobacteria both *in vivo* and *in vitro* [Snart *et al.* 2006, Jaskari *et al.* 1998, Drzikowa *et al.* 2005, Mitsou *et al.* 2010].

In vitro studies. In an earlier study, the ability of different bacterial strains to degrade oligomers prepared from oat β-glucan, xylan, and raffinose, was analysed *in vitro*. While raffinose stimulated the probiotic strains significantly, only *L. rhamnosus* GG was shown to consume over 70% of the total β-glucan oligomers in the growth medium, after 24h incubation. The other bacterial strains utilised β-glucan oligomers poorly. The overall digestion of *L. acidophilus* strain was 17% and *Bifidobacterium* strains 4% [Jaskari *et al.* 1998].

In another study, β-glucan was evaluated among 11 different types of commercially available carbohydrates and compounds for its ability to enhance the growth of *Lactobacillus acidophilus*, *Bifidobacterium animalis lactis* and *Lactobacillus casei* cultures. B-glucan hydrolysate enhanced the growth of *B. animalis* more than inulin supplementation. Also, both forms of β-glucans tested, hydrolysate and concentrate, increased the growth of *L. casei*. However, they could not support the growth of *L. acidophilus* [Su *et al.* 2007].

Both barley and oat β-glucan addition was shown to support the growth and viability of *B. animalis lactis* in a yogurt during prolonged cold storage. The mixed yoghurt culture, *L. bulcaricus* and *S. thermophilus*, were unaffected by β-glucan addition [Vasiljevic *et al.* 2007].

In a study regarding the *in vitro* fermentation of oat and barley β-glucan (representing different molecular weight) inoculated with human faecal samples, where changes in microbiota and SCFA production were monitored, a significant increase in *Clostrium histolyticum* subgroup and *Bacteroides-Prevotella* group was recorded. The β-glucan oligosaccharide fraction with a low degree of polymerization (3-4 oligomers) significantly increased the *Lactobacillus-Enterococcus* group. In general, β-glucans did not exert prebiotic properties. The SCFA profile was propionate rich [Hughes *et al.* 2008].

In a more recent study, the effect of β-glucans enriched pasta and barley flour on the growth and probiotic features of four *Lactobacillus* strains was investigated. The ability of β-glucans to influence the growth of microorganisms was strain-selective, as it has been pointed out, several times before [Arena *et al.* 2014].

Animal studies. The effects of cereal β-glucan on intestinal microbiota of rats were tested. After 3, 6 and 7 weeks of β-glucan administration, the rats exhibited higher *Bifidobacterium* and *Lactobacillus* and significant lower *Enterobacteriaceae* counts compared to the control group.

The influence still existed after the end of cereal β-glucan administration. High dose β-glucan was more effective than low dose β-glucan in modifying intestinal microbiota and also the oat β-glucan improved intestinal biota more efficiently compared to barley β-glucan at the same dose, propably due to oat β-glucan's higher percentage of insoluble fibre [Shen *et al.* 2012].

In another animal study the effect of oat consumption was tested in the intestinal microbiota of rats. Different oat based diets were given to different groups of rats, while the control group received an oat-free diet. In most test groups, counts of bifidobacteria were significantly higher and counts of coliforms were significantly lower compared to control. In all the oat-based diet groups, pH values were significantly lower in the intestinal contents, and caecal concentrations of acetate, propionate, butyrate, and total SCFA were significantly higher [Drzikova *et al.* 2005].

In an effort to examine the prebiotic effect of different sources of dietary fibre, rats were given casein-based diets supplemented either with barley flour, oatmeal flour, cellulose, or high- or low-viscosity barley β-glucan. High viscosity (HV) barley β-glucan containing diet resulted in large and highly consistent difference in microbiota compared to other diets. Measurements of *L. acidophilus* showed that lactobacilli formed a greater proportion of the cecal microbiota in HV barley β-glucan-fed rats compared to cellulose-fed animals.This result confirmed that some lactobacilli utilize oligosaccharides present in β-glucan hydrolysates [Snart *et al.* 2006].

Human studies.The prebiotic potential of low-dose barley β-glucan was evaluated on 52 healthy volunteers. In older subjects (≥50 years old), β-glucan supplementation induced a strong bifidogenic effect significantly increasing the bifidobacteria counts. An increase was also recorded in bacteroides counts in this age group [Mitsou *et al.* 2010].

In another study, the total faecal microbiota and metabolomes of 26 healthy subjects before and after a dietary intervention with durum wheat flour and whole-grain barley pasta were compared. Two months intervention resulted in higher levels of *Clostridiaceae* (*Clostridium orbiscindens* and *Clostridium* sp.), *Roseburia hominis*, and *Ruminococcus* sp. and lower levels of other *Firmicutes* and *Fusobacteria* compared to baseline faecal samples. Lactobacilli increased, while the numbers of Enterobacteriaceae, total coliforms, and *Bacteroides, Porphyromonas, Prevotella, Pseudomonas, Alcaligenes,* and *Aeromonas* decreased. A marked increase in SCFA, such as 2-methyl-propanoic, acetic, butyric, and propionic acids, was also recorded after the intervention [De Angelis *et al*. 2015].

6.2 Role of cereal β-glucan in colon cancer prevention and/or treatment

In CRC prevention the most studied β-glucans such as lentinan, grifolan, scleroglucan and schyzophyllan are extracted from mushrooms. Their anticarcinogenic effects are demonstrated

on both animals and humans studies. These β-glucans have reported to mediate their anti-tumour activity by activating the host's immune system, via activation of leukocytes and production of inflammatory cytokines, by inhibiting tumour growth, metastasis, and the development of precancerous ACF lesions in the colon [Sveinbjornsson *et al.* 1998, Xiao *et al.* 2004, Kimura *et al.* 2006, Bobek and Galbavy 2001]. B-glucans have been used as adjuvant therapy in clinical trials with a positive effect on patients' survival and quality of life. Mushroom β-glucan administration to colon cancer patients undergoing chemotherapy resulted in prolonged survival compared to a control group [Wakui *et al.* 1986], limited the side effects of chemotherapy, and improved the quality of life overall [Hazama *et al.* 2009].Seaweed and yeast derived β-glucans have also been investigated for their potentiality to treat cancer. In colorectal cancer patients they have the ability to reduce adverse effects of chemotherapy, such as leukopenia, oral mucositis and diarrhoea [Karaca *et al.* 2014], and to reduce the incidence of severe post-operative infections and complications after high-risk gastrointestinal operations [Dellinger *et al.* 1999]. In rats, as well as shortening the recovery of leukopenia they have expressed the capacity to increase antibody formation [Větvička *et al.* 2007].

Recently, the anticancer properties of cereal β-glucans have been under intensive investigation. Data from several *in vitro* studies show that cereal β-glucans can prevent CRC initiation by reducing FW genotoxicity [Angeli *et al.* 2009, Oliveira *et al.* 2006, Zimmermann *et al.* 2015]. The mechanism is not yet known but they may act as antioxidants [Kofuji *et al.* 2012, Angeli *et al.* 2009, Oliveira *et al.* 2006] or immunomodulators.

Oxidative stress is considered to be one of the primary causal factors for various diseases and aging [Kofuji *et al.* 2012], plays an important role in CRC development while free-radicals trapping antioxidants are important for the treatment and prevention of CRC [Chang *et al.* 2008]. Barley β-Glucan exhibits antioxidant activity significantly higher than that of various polymers that are used as food additives. This activity is influenced by different physiologic properties (e.g., structure and molecular size) of β-glucan, which varies depending on the source and extraction method used [Kofuji *et al.* 2012].

Immunomodulatory activity after oral administration of cereal β-glucans is suggested to result primarily from ingestion of small particles of β-glucan by pinocytic M cells located in Peyer's patches of the small intestine [Di Luzio *et al.* 1970]. Once stimulated, these cells can migrate to the lymph nodes and are capable of activating other macrophages, NK cells, and T lymphocytes via the release of cytokines [Sakurai *et al.* 1992, Suzuki *et al.* 1989]. Cereal β-glucans have been shown to interact with immune cells, stimulating the immune system directly [Slavin 2013]. In two older animal studies, oral administration of β-1,3/1,4-glucan from barley to mice resulted in the activation of antitumor monoclonal antibodies, leading to enhanced

tumourregression and survival through a mechanism involving the macrophages [Hong *et al.* 2004, Cheung *et al.* 2002]. Interestingly, suppression of tumour growth again was highly correlated with β-glucan molecular weight [Cheung *et al.* 2002].

Data from recent studies show that cereal β-glucans also have the ability to inhibit cell proliferation, to alter gene expression, or to act as a radioprotective agent in cancer cell lines [Choromanska *et al* 2015, Parzonko *et al.* 2015, Jafaar *et al.* 2014, Ghavami*et al.* 2014]. Antitumor activities of low molecular weight β-glucan derived from oats were investigated in cancer cells (Me45, A431 and normal HaCaT and murine macrophages P388/D1). Oat β-glucan significantly deceased cancer cells viability, while it was non-toxic for normal cells. Immunocytochemical analysis showed that β-glucan induced strong expression of caspase-12 in both cancer cell lines, while in HaCaT cells ABC reaction was significantly lower and in P388/D1 cell line ABC reaction was negative [Choromanska *et al.* 2015]. In another study the inhibitory effect of (1,3)(1,4)-β-D-glucan from oat was explored on human skin melanoma HTB-140 cells. The oat β-D-glucan caused an increase of caspase-3/-7 activation and an appearance of phosphatidylserine, demonstrating the induction of apoptosis. Intracellular ATP level decreased along with the mitochondrial potential suggesting a mitochondrial pathway of apoptosis. The results from a cell cycle analysis showed increase in the number of apoptotic cells, an increase in the number of cells in G1 phase and a decrease in the number of cells in G2/M [Parzonko *et al.* 2015]. The activity of β-D-glucan from barley was tested in endocrine-sensitive MCF-7 versus endocrine-resistant LCC9 and LY2 breast cancer cells. Barley-derived β-glucan inhibited endocrine-resistant breast cancer cell proliferation and altered gene expression [Jafaar *et al.* 2014]. Barley β-glucan can enhance radioprotection in the human hepatoma cell line HepG2. Pre-treatment with β-glucan, 1μg/mL, for 72h protected HepG2 cells against radiation, as indicated by increased surviving fraction, reduced apoptosis, and fewer DNA strand breaks [Ghavami *et al.* 2014].

7. Possible indicators for colon cancer prediction

7.1 Short-chain fatty acids (SCFA) concentration

Functional foods, such as prebiotics and probiotics, dietary fibres, and other dietary components are known to enhance short chain fatty acid (SCFA) production in the colon. The SCFAs, primarily butyrate, acetate, and propionate, are produced by colonic bacteria fermentation of dietary carbohydrates, specifically resistant starches and dietary fibre. The rate and amount of SCFA production depends on the species and amounts of bacteria present in the colon, the substrate source, and gut transit time [Wong *et al.* 2006]. Specific SCFA may reduce the risk of

developing gastrointestinal disorders, cancer, and cardiovascular disease [Hijova *et al.* 2007]. Acetate is detected in higher amounts in faeces, with propionate being the second and butyrate the third (acetate > propionate ≥ butyrate) [Topping and Clifton 2001] with a molar ratio 60:20:20 [Cummings *et al.* 1979].

Acetate (C2), the principal SCFA in the colon, is readily absorbed and transported to the liver. Therefore, it is less metabolized in the colon [Cook and Selling 1998] and is the main SCFA in the blood. Acetate is the main substrate for cholesterol synthesis. It has been shown to increase cholesterol synthesis after absorption [Wong *et al*. 2006].

Propionate (C3) is largely taken up by the liver. It is a substrate for hepatic gluconeogenesis and has been reported to inhibit cholesterol synthesis in the hepatic tissue [Venter *et al.* 1990]. Increased production of propionate may inhibit hepatic cholesterol synthesis, but there is not enough data from human trials [Wong *et al.* 2006].

Butyrate (C4) is the most important SCFA for colonocyte metabolism being their major energy source. Butyrate regulates colonocyte metabolism, controlling their motility and water and electrolyte absorption. It also protects the cells against infections and inflammatory onsets [Celasco *et al.* 2014]. It is estimated that 70-90% of bacterially produced butyrate is metabolized by the epithelium and it is used by colonocytes preferentially over propionate and acetate in a ratio 90:30:50 [Cummings 1984]. The most important butyrate producers are *Faecalibacterium prausnitzii*, *Clostridium leptum*, *Eubacterium rectale*, and *Clostridium coccoides* [Louis and Flint 2009].

Butyrate has many other important and protective functions, being a regulator of mucosal gene expression, differentiation and apoptosis [Thomas *et al.* 2014, Zhang *et al.* 2010, Roy *et al.* 2009, Kopp *et al.* 2009, Hinnebusch*et al.* 2002]. Data from various studies suggest that butyrate may also protect against CRC and colitis [Celasco *et al.* 2014, Maciа*et al.* 2012, Zhang *et al.* 2010, Roy *et al.* 2009, Kopp*et al.* 2009]. One possible mechanism is butyrate's ability to support the regeneration of the intestinal epithelium. Metagenomic data from a study by Vital *et al.* revealed genomes of 225 bacteria with a potential to produce butyrate. The majority of candidates belong to distinct families within the Firmicutes, but members of nine other phyla, especially from Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, Spirochaetes, and Thermotogae, were also identified as potential butyrate producers [Vital *et al*. 2014].Acetate and propionate have also been shown to induce apoptosis in human colon cancer cell lines but to much lesser degree than butyrate [Hinnebusch *et al.* 2002, Hague *et al.* 1995].

Although butyrate is widely studied and established for its anticarcinogenic properties, a variety of other potential SCFAs also exist in the colon. Valerate (C5) and the branched chain fatty acids iso-butyrate (iC4) and iso-valerate (iC5), are metabolites from bacterial degradation

of protein. Whereas the unbranched SCFA butyrate induces apoptosis, two derivatives of butyrate, iso-butyric acid and heptafluorobutyric acid, are ineffective in inducing either differentiation or apoptosis [Heerdt *et al.* 1994]. Valerate together with propionate caused growth arrest and differentiation in human colon carcinoma cells (HT-29, HCT-116) [Hinnebusch *et al.* 2002]. The ability of butyrate, propionate and valerate to inhibit human colon carcinoma cell proliferation and to induce apoptosis *in vitro* was recently investigated in detail. It was discovered that each SCFA possessed specific effects; butyrate was shown to have more pronounced effects on the keratins and intermediate filaments (IFs), while valerate altered the β-tubulin isotypes' expression and the microtubules (MT, cellular structures that help move chromosomes during mitosis). Propionate was involved in both mechanisms displaying intermediate effects. Valerate was found to show a potential antimicrotubule agents activity. An antimicrotubule agent is a type of drug used to treat cancer. It blocks cell growth by stopping mitosis through interfering with MTs [Kilner *et al.* 2012 and 2016].

Caproate (C6) and heptanoate (C7) belong to the group of medium-chain fatty acids (MCFAs). The origin of caproate (C6) is less known, but there is evidence suggesting it may belong to the protein-derived group together with the other minor acids [Siigur *et al.* 1994]. The anticancer properties of capric, caprylic, and caproic acids on human colorectal, skin and mammary gland cancer cells was investigated and caproate reduced cancer cell viability by 70% to 90% compared to controls, exhibiting a potential anticancer effect [Narayanan *et al.* 2015]. In another study using human colorectal cancer cells, caproate failed to have any appreciable effects on cell growth or differentiation [Hinnebuchs *et al.* 2002].

A 6 weeks administration of cereal β-glucan increased colonic SCFA in rats. There was a statistically significant increase in the total SCFA concentrations in both the low-and high-dose oat β-glucan group, and in the high-dose barley β-glucan group compared to the control group, whereas no significant differences were observed in total SCFA concentrations in the low-dose barley β-glucan group compared to the control group [Shen *et al.* 2012]. There was no significant change in butyrate concentration, although both high-doses of oat and barley β-glucan tended to increase it. Oat β-glucan tended to be more effective in increasing the total SCFA concentrations than barley β-glucan, but the difference was not significant [Shen *et al.* 2012]. Also, ingestion of oat derived β-glucan has been shown to increase butyrate concentration [Queenan *et al.* 2007, Drzikova *et al.* 2005].

7.2 Bacterial enzyme activity

The risk for CRC has been associated with several intestinal bacterial enzyme activities [Nakamura *et al.* 2002]. From these faecal bacterial enzymes β-glucuronidase, β-glucosidase, nitroreductase, and azoreductase have been extensively studied.

Hydrolysis of glycosidic bonds is one of the best-known examples of bacterial metabolism. Glycosides in the gut originate either from the diet or are excreted by the liver via the bile [de Preter *et al.* 2008]. The intestinal microbiota can hydrolyse the glycosidic bond resulting in the release of active aglycons, some of which are potentially toxic or carcinogenic [Takada *et al.* 1982]. The principal glycosidases produced by the intestinal microbes are β-glucuronidase and β-glucosidase. Response to different diets has been mainly investigated for these two enzymes. They are also being discussed as possible biomarkers for colorectal cancer risk, as they have the potential to liberate carcinogenic metabolites [McIntosh *et al.* 2012]. Little is known to date on whether β-glucosidase and β-glucuronidase activities vary in response to changes in dietary intakes or to the exposure to dietary glycosides reaching the colon [McIntosh *et al.* 2012].

β-glucuronidase. β-glucuronidase is generated by a wide range of gut bacteria including *Escherichia coli*, and some species of *Bacteroides* and *Clostridium* [Gloux *et al.* 2011]. This enzyme interferes with the detoxification process by uncoupling glucuronides and consequently deconjugating potential toxins. It cleaves β-glucuronic acid linkages from the non-reducing termini of glycosaminoglycans such as chondroitin, sulfate, heparan sulfate, and hyaluronic acid [Paigen 1989]. β-glucuronidase has a role in enterohepatic circulation of various drugs, hormones and toxins, and modification of this enzyme activity has been shown to affect the metabolism and tumorigenicity of carcinogens [Takada *et al.* 1982, Kuhn 1998, LoGuidice *et al.* 2012]. Furthermore, a correlation has been found between elevated levels of β-glucuronidase and an increased risk of colon cancer and that excessive β-glucuronidase activity may be a prime factor in the aetiology of colon cancer [Kim and Jin 2001, Gill and Rowland 2002, Rafter *et al.* 2004]. While measuring β-glucuronidase activity in colon cancer patients and healthy controls, it was discovered that the faecal β-glucuronidase activity of patients with colon cancer was 12.1 times higher than that of the healthy controls [Kim and Jin 2001]. Diet plays a role in β-glucuronidase activity. Obese volunteers on the high-protein/moderate-carbohydrate weight-loss diet showed an increase in faecal bacterial β-glucuronidase activity [McIntosh *et al.* 2012]. A high-risk diet for colorectal cancer significantly increased β-glucuronidase activity, while β-glucosidase activity did not show a strong relationship with high-risk diets [Rafter *et al.* 2004].

β-glucosidase.β-glucosidase is a glucosidase enzyme located in the brush border of the small intestine. Among other bacteria, lactobacilli and bifidobacteria show high β-glucosidase activity [Saito *et al.* 1992, Rowland and Tanaka 1993, Otieno *et al.* 2005]. It belongs to a heterogeneous group of exo-type glycosyl hydrolases that cleave β-glucosidic linkages in disaccharide or glucose-substituted molecules [Jeng *et al.* 2010]. β-glucosidases play fundamental biological roles in processes such as the degradation of cellulose and other carbohydrates for nutrient uptake [Beguin 1990, Bhatia *et al.* 2002]. Several studies have shown that oligosaccharides have the ability to increase the activity of this enzyme, and this may be attributed to the ability of oligosaccharides to stimulate the growth of lactic acid bacteria [Saito *et al.* 1992, Rowland and Tanaka 1993]. β-glucosidase produced by probiotics (mainly by lactobacilli) has been found to prevent colon cancer by releasing flavonoids with anticarcinogenic, antioxidative, antimutagenic, and immune stimulatory effects [Wollowski *et al.* 2001].

Enzyme activity and pre- and/or probiotic intake. There are several studies on the influence of different pre- and probiotics and/or their symbiotic combination on faecal bacterial β-glucuronidase and β-glucosidase activity. In general, supplementation with either lactobacilli or bifidobacteria decreases β-glucuronidase [Verma and Shukla 2013, de Preter *et al.* 2008, Bouhnik *et al.* 1996a] and increases β-glucosidase activity [Verma and Shukla 2013, de Preter *et al.* 2008]. Usually this effect is accompanied by a reduction in bacterial species with high β-glucuronidase activity, such as *Bacteroides* spp. and *Clostridium* spp. [Molan *et al.* 2014, de Preter *et al.* 2008]. It has been also reported that bifidobacteria and lactobacilli in comparison with other bacteria possess high levels of β-glucosidase [Rowland and Tanaka 1993, Verma and Shukla 2013, Otieno *et al.* 2005, Saito *et al.* 1992] and low levels of β-glucuronidase activity [Nakamura *et al.* 2002, McConnell and Tannock 1993].

The effect of lactulose, oligofructose-enriched inulin, *L. casei Shirota*, *B. breve* and *S. boulardii* on faecal β-glucuronidase and β-glucosidase activity was investigated in a randomized, crossover study in 53 healthy volunteers. Lactulose and oligofructose-enriched inulin significantly decreased β-glucuronidase activity, whereas a tendency to decrease β-glucuronidase activity was observed after *L. casei Shirota* and *B. breve* intake. As expected, *B. breve* ingestion increased β-glucosidase levels, but did not affect β-glucuronidase activity. Supplementation with the synbiotic did not appear to be more effective than either compound alone [de Preter *et al.* 2008].

The effects of *L.*GG, *L. casei*, *L. acidophilus*, *L. plantarum* and *B. bifidum* on faecal enzymes and preneoplastic ACF formation in early colon carcinogenesis was studied in rats. Treatment with L.GG, and *L.acidophilus* significantly decreased β-glucuronidase activity and increased

β-glucosidase activity during the intervention whereas treatment with *B. bifidum* significantly decreased β-glucosidase activity [Verma and Shukla 2013]. The unexpected decrease in β-glucosidase activity by *B. bifidum* could be explained by the higher β-glucosidase activity in lactobacilli compared to bifidobacteria [Verma and Shukla 2013, Otieno *et al.* 2005, Saito *et al.* 1992].

The effect of Australian sweet lupin kernel fibre on colon cancer markers was examined in a crossover study in healthy men. Among other variables measured (butyrate concentration and output, frequency of defecation, faecal output, moisture, transit time and pH) 1-month consumption of fibre from lupins resulted in a significant decrease of β-glucuronidase activity compared to the control diet [Johnson *et al.* 2006].

Results are not always convincing. A 3-week administration of probiotic fermented milk and inulin increased the faecal content of lactobacilli and bifidobacteria compared to baseline but did not affect either β-glucuronidase or β-glucosidase activity [Sairanen *et al.* 2007]. In a study on human *in vitro* gut bacterial ecosystems-model investigating the metabolism of inulin and GOS with respect to bacterial growth and anti-mutagenicity potential, a decrease was recorded in both enzymes. Inulin metabolism, after stimulating the growth of lactobacilli, peptostreptococci, enterococci, and *Cl. perfringens* populations, had a minor reduction in β-glucosidase and β-glucuronidase activity, while GOS metabolism after increasing lactobacilli (was only weakly bifidogenic) strongly suppressed both β-glucosidase and β-glucuronidase activity [McBain and Macfarlane 2001].

Enzyme activity and cereal β-glucan intake. The effect of β-glucan on these enzymes activity has not yet been studied in humans. The effect of oat and barley β-glucan on faecal characteristics, intestinal microbiota, and intestinal bacterial metabolites was studied in rats. Among other metabolites, cereal β-glucan administration (both oat and barley β-glucan) induced a significant decrease in β-glucuronidase activity in a dose-dependent manner. Concurrently, the population of *Lactobacillus* and *Bifidobacterium* increased, whereas the number of *Enterobacteriaceae* decreased [Shen *et al.* 2012].

7.3 Faecal moisture

Faecal waste consists of approximately 75% water, and 25% of solid matter, and this percentage may vary according to individual and the residence time of the faeces in the intestine. It may also include dead bacteria (help us to digest food), fibre (undigested food debris), protein, cellular linings, salts, fats, substances released from the liver and intestines (such as mucus). Unhealthy faeces may have more than 90% moisture content and this is an indication of diarrhoea.

Constipation (abdominal discomfort, only one or two bowel movements per week, and hard stool consistency) may cause the faeces to become extremely hard. Faecal moisture is shown to be increased with high fibre diets [Johnson*et al.* 2006, Takahashi 1994]. Increased faecal moisture leads to increased transit time and has beneficial effect on chronic constipation.

Cereal β-glucan administration significantly increased faecal moisture compared to control in rats. After stopping cereal β-glucan administration for one week, the faecal moisture of cereal β-glucan groups, besides the low-dose barley β-glucan group (0.35 g/kg of body weight), was still higher than that of the control group. In this study oat β-glucan was more efficient in terms of increasing the faecal moisture in rats than barley β-glucan [Shen *et al.* 2012].

7.4 Faecal pH

A-high colonic pH is believed to promote co-carcinogen formation from bacterially degraded bile acids or cholesterol, therefore promoting colorectal cancer. This procedure is inhibited at a faecal pH lower than 6.5 [Thornton 1981]. Subjects with CRC tend to have higher faecal pH than those with adenomas or with no colorectal disease [Thornton 1981, Ishikawa *et al*. 2005, Ohigashi *et al.* 2013]. Increased carbohydrate consumption reduces the pH of gut lumen, which likely plays a major role in determining bacterial metabolism and competition [Flint 2007].

The effect of SCFAs propionate and acetate and pH was studied on human colon cancer HT-29 cells. The goal was to examine the precise mechanisms, the kinetics of cellular events and the impact of environmental factors such as pH. The acidic pH, ranging from 6 to 7.5 led cancer cells from apoptosis to necrosis after the exposure to propionate and acetate (a process time lasting more than 96 h). In contrast, when the pH was 5.5, the same acids induced a much more rapid and efficient cell death within less than 24h [Lan *et al.* 2007].

The effect of FOS and inulin intake on faecal bifidobacteria and selected metabolites involved in colonic carcinogenesis was studied in the context of two clinical studies. Even though bifidobacteria counts were significantly increased during the interventions they recorded no significant effect on faecal pH, or on the activities of bacterial nitroreductase, azoreductase, and β-glucuronidase enzymes. However, the study populations were relatively small (12 and 20 subjects) and intervention lasted only for 12 days [Bouhnik *et al.* 1996a+b]. When faecal risk factors for colon cancer in 38 healthy men were examined, a 1-month administration of lupin kernel fibre food diet among other effects significantly decreased the faecal pH [Johnson *et al.* 2006]. Finally, in an oat β-glucan and barley β-glucan intervention study on rats, as the population of *Lactobacillus* and *Bifidobacterium* increased and the number of *Entero-bacteriaceae* decreased, faecal pH values for each cereal β-glucan group significantly decreased in comparison to the control group [Shen *et al.* 2012].

7.5 Faecal water (FW) genotoxicity

The relationship between diet and the aqueous phase of human faeces has been widely studied. FW activity markers are believed to be affected by specific dietary components associated with CRC risk [Pearson *et al.* 2009]; a high risk diet, a diet high in animal fat, protein, and sugar and low in dietary fibre induced almost twice as high FW genotoxicity compared to low risk diet (a diet low in animal fat, protein, and sugar and high in fibre) [Rieger *et al.* 1999]. Other components linked to decreased FW genotoxicity are calcium [Wang *et al*. 2010], probiotics, and prebiotics [Rafter *et al.* 2007, Burns and Rowland 2004, Klinder *et al.* 2004].

FW contains bioactive compounds originated from diet such as bile acids, fatty acids, N-nitroso compounds, heterocyclic amines, which are potentially related to CRC initiation and development. It could be thus proposed as an additional early biomarker, given that it can be collected easily in a non-invasive manner. Genotoxicity of FW is one of the biological activities that have been widely studied in colonic cell lines using the single cell gel electrophoresis (COMET) assay [Pearson *et al.* 2009]. Human colon cells are often used as target cells because they are considered to be surrogates of the human colon epithelium *in vivo* [Venturi *et al*. 1997].

Prebiotics, probiotics, and their symbiotic combinations have been tested for their ability to decrease FW genotoxicity. In a recent study the effect of wheat bran extract (WBE) on the markers of CRC risk was evaluated in healthy volunteers. WBE stimulated selectively the growth of *Bifidobacterium adolescentis* and altered the colonic fermentation pattern and significantly reduced colonic protein fermentation compared with the run-in period. However, FW cytotoxicity and genotoxicity were not affected in this study [Windey *et al.* 2015].

The effect of FOS enriched inulin, *L.rhamnosus* GG and *B.lactis* Bb12 and their synbiotic preparation, on the genotoxicity of FW of polypectomised or colon cancer patients was examined. The DNA-damaging capacity of FW was analysed by using human colon cancer HT-29 cells. Reduced genotoxicity was observed only on polypectomised patients and after consuming the synbiotic diet [Rafter *et al.* 2007].

The antigenotoxic properties of different probiotic strains (*Bifidobacterium* Bb12, *Bifidobacterium* sp. 420, *Lactobacillus plantarum, L. bulgaricus, Enterococcus faecium*) were examined against genotoxic challenge of FW on human colon cancer HT-29 cell lines. Genotoxicity was reduced by all bacteria strains, the highest change resulting from incubation with *Bifidobacterium* Bb12 and *Lactobacillus plantarum*. In a second series of experiments the bacteria strains were cultured in FOS-based prebiotics (Inulin, Raftiline, Raftilose, and Actilight), galacto-oligosaccharides (Elixor), or maltodextrin (Fibersol). The HT-29 cells were incubated in the presence of supernatant from each culture before exposure to genotoxic faecal water. Their resistance to the DNA changes caused by the FW was measured and the most

effective protective combination was the culture of *Lactobacillus plantarum* in FOS-based prebiotics [Burns and Rowland 2004].

Similar results were gained while examining the effect of inulin/oligofructose and *Lactobacillus rhamnosus* and *Bidobacterium lactis* on the genotoxicity in HT-29 cells and on AOM-treated tumor-free rats. An inulin-based diet reduced both faecal and caecal genotoxicity [Klinder *et al.* 2004].

In vitro chemoprotective activity of cereal β-glucan has been reported against chemical mutagens although in different cell lines. Antigenotoxic and anticytotoxic effects of barley β-glucan were seen in human hepatic cell HepG2 [Angeli *et al.* 2009], in hamster ovarian cell CHO-k1 and rat hepatic HTC cell lines [Oliveira *et al.* 2006]. Furthermore, barley β-glucan was found to be dose-dependently both geno- and cytoprotective in broiler chicken lymphocytes exposed to increasing concentrations of aflatoxin B1 [Zimmermann *et al.* 2015].

Consumption of cereal β-glucan may also have an immunostimulating effect through FW. Effect of oat β-glucan on enterocytes (INT407, Caco-2, HT29, and T84) was studied; 6 ileostomic patients consumed a control diet or an oat β-glucan enriched diet (5 g). The FW was prepared from ileostomic content, and added to cell lines together with a cytokine mixture (IL-1β + INFγ + TNFα). The incubation with β-glucan enriched FW significantly increased IL-8 production in HT29 and INT407 cells, and intercellular adhesion molecule (ICAM)-1 expression in T84 and Caco-2 cells compared to placebo. These results indicated that consumption of oat β-glucan had an immune-stimulating effect [Ramakers *et al.* 2007].

7.6 Body mass index (BMI)

An increasing number of diseases in the western world, such as cancer, are related to lifestyle, physical inactivity, obesity, smoking, and alcohol consumption. High caloric intake and reduced activity are the main contributors to the development of metabolic syndromes, and genetic predispositions are also increasing the risk. A high BMI and waist circumference are risk factors for CRC, although little is known about the connection between these parameters and the different molecular disease subsets. Several studies have demonstrated that childhood and adolescent height and weight play a role inCRC development; energy restriction at a young age decreased the risk of CRC later in life [Hagland and Søreine 2014].

Effect of cereal β-glucan on body weight was investigated among other indicators of CRC on rats. During the study the weight gain in the control group was higher than that in the β-glucan groups; even though the body weight of the oat β-glucan oral-administration group was significantly lower than that of the barley β-glucan group. These results suggest that both oat and barley β-glucans might play a potential role in weight control or weight loss [Shen *et al.* 2012].

7.7 Age

The composition of the intestinal microbiota co-develops with the host from birth and is subject to a complex interplay involving genetics, diet, and lifestyle [Nicholson *et al*. 2012, Clark *et al.* 2015]. Aging is a major risk factor for age-related inflammatory processes and diseases, including cachexia, frailty, cancer, and metabolic as well as neurological diseases resulting from 'inflamm-aging' [Bischoff 2016]. According to the data from several studies, the microbiota of older people differs from that of younger adults [Claesson *et al*. 2011] and microbiota composition in the elderly correlates with measures of frailty, comorbidity and inflammation [Claesson *et al.* 2012]. CRC incidence is strongly related to age, with the highest incidence rates being in older men and women. In the UK between 2010 and 2012, an average 43% of CRC cases were diagnosed in people aged older than 75 years, and 95% were diagnosed in those aged older than 50 [Cancer research UK 2015].

Microbiotas of populations over 60 years of age have often an altered bacterial composition towards that of a more proteolytic one. This change consists of an increase in proteolytic bacteria and a decrease in saccharolytic bacteria [Bischoff 2016, Mäkivuokko *et al.* 2010, Woodmansey 2007]. Known butyrate producers, such as *Clostridium* cluster XIVa and *Faecalibacterium prausnitzii*, have been observed in lower levels in older volunteers [Mueller *et al.* 2006, Rajilic-Stojanovic *et al.* 2009]. These changes are of consequence in regard to morbidity and mortality in the elderly population, though use of probiotics, and/or prebiotics and other lifestyle interventions might delay, or even reverse, such alterations [Bischoff 2016].

Moreover, the number and diversity of the health-positive bifidobacteria are shown to reduce in the elderly [Hopkins *et al*. 2001, Hopkins and Macfarlane 2002]. These changes coincide with an increased risk of disease development and possibly could be prevented by prebiotics consumption because of their ability to increase bifidobacteria counts. For this group of bacteria recent studies on prebiotics have been conducted regarding their ability to reverse the age-related decline in bifidobacteria and to modulate associated health parameters [Vulevic *et al.* 2015, Walton *et al.* 2012].

7.8 Physical inactivity

Lack of exercise is an identified risk factor for cancer development and mortality in several site-specific cancers, such as colorectal cancer [Colditz *et al.* 1997, Lee *et al.* 2012], lung cancer [Bade *et al.* 2015, Murphy *et al.* 2004] and breast cancer [Lahart *et al.* 2015]. It is suggested that exercise and physical activity should be used as an important therapeutic option in cancer treatment. Exercise can reduce symptoms, increase tolerance, improve quality of life, and potentially reduce length of stay and postoperative complications [Bade *et al.* 2015].

A literature review of 23 case-control (cross-sectional) and 17 cohort (longitudinal) studies on exercise and prevention or treatment of chronic diseases, such as CRC, shows conclusive evidence that physical activity primarily prevents or delays chronic diseases [Booth *et al.* 2012]. In one study by Colditz *et al.*, those in the highest physical activity category had approximately 40 - 50% reduction in risk of colon cancer compared with the least active category [Colditz *et al*. 1997].

The possible mechanisms through which physical inactivity might increase prevalence of colon cancer are: (1) lengthening the transit time of faeces, thus prolonging exposure to faecal carcinogens; (2) causing higher levels of blood insulin, thus producing insulin resistance, which is a risk factor for cancer; (3) causing higher levels of blood free IGF-I, exposing the rapidly turning over colon epithelium to higher levels of anabolic hormone associated with greater colon cancer incidence; (4) preventing the synthesis and release of exercise-derived, anti-inflammatory myokines, thus removing their systemic effect; and/or (5) producing positive energy expenditure, increasing body fat [Booth *et al.* 2012].

Animal studies have focused on the inhibiting role of exercise in the incidence and progression of cancer and in tumour metastases. The independent and combined effects of short-term moderate-exercise training and oat fibreβ-glucan on the metastatic spread of injected tumour cells and macrophage antitumor cytotoxicity were investigated. Mice were assigned to one of four groups: (1) exercise, (2) β-glucan, (3) exercise together with β-glucan, and (4) control. B-glucan was fed in the drinking water for 10 days before tumour administration and death. Exercise consisted of treadmill running (1 h/day) for 6 days. Both moderate exercise and β-glucan administration decreased lung tumour foci and increased macrophage cytotoxicity to melanoma [Murphy *et al.* 2004].

8. Aim

The aim of this study was:

(1) to investigate the effect of barley-derived β-glucan on the gut microbiota of polypectomised patients (*in vivo*) taking into account parameters such as faecal microorganisms, the concentration of faecal short-chain fatty acids, faecal bacterial enzymes, stool pH, and moisture.

(2) to examine whether β-glucan consumption by polypectomised patients has as effect on their faecal water (FW) *in vitro* genotoxicity (*in vitro*).

9. Materials and methods

9.1 Study place

This study was conducted in collaboration of Harokopio University, (Department of Nutrition and Dietetics, Laboratory of Biochemistry, Molecular Biology and Microbiology) and the 2nd Department of Gastroenterology, Evangelismos Hospital (Director: D.C. Karamanolis), the Department of Gastroenterology, "Saint Panteleimon" General Hospital, Nikea (Director: I.K. Triantafillidis) and the Anti-Cancer Hospital Agios Savas, Athens, Gastorenterological clinic B (Director: D. Dimitroulopoulos). The protocol was approved by the Bioethics Committee of Harokopio University (20/29-05-2008).

9.2 Subjects

Adults were recruited after having histologically confirmed adenomatous polyps 1cm or more in size or alternatively three or more polyps with low to severe dysplasia.

Exclusion criteria were: age ≥75 years, pregnancy or desire to become pregnant during the study period, subjects who were considered to be poor clinic attendees, subjects who had been on antibiotics within the previous two months or were likely to require antibiotics during the trial, subjects who had consumed prebiotics or probiotics within the previous month or were likely to require prebiotics or probiotics during the trial and subjects with colon cancer, additional gastrointestinal disorders (eg. Chron's disease or ulcerative colitis), malignancy or any end-stage organ disease.

The volunteers were divided randomly into two groups. One group was given the experimental food (β-glucan group) and the other a placebo (control group). The study was conducted as a double-blind randomized clinical trial. Randomization was done by using algorithm generated random number, e.g. 1,1,0, 1,1,0... where patients categorized as 1 ate the experimental food while those categorized as 0 received a placebo.Neither the patient nor the

research team knew the results of the randomization. Study participants provided their stool sample 2 weeks after their colonoscopy preparation. This period of time has previously been shown to be sufficient to allow the microbiome to recover [O'Brien *et al.* 2013].

On a study day subjects collected their faeces from one total passage in an aerobic container, from which ~5g of faeces was removed and kept in an anaerobic container for microbiological analysis. Faecal volume and pH were recorded and a portion of the total passage was kept in the freezer until analysis of faecal bacterial enzyme activity (β-glucuronidase and β-glucosidase) and faecal SCFA concentration. The remainder of the total passage was centrifuged to collect the faecal water, which was then kept at -80^{o}C until further analysis.

9.3 Dosage /Feeding regime

The placebo food was bread prepared using commercially available flouraccording to the following recipe (for one bread): wheat flour 500g; olive oil 1.5 tbsp.; dry baking yeast 40g; sugar 1 tbsp.; salt 1 tsp; water 370 mL. After baking, each bread was divided into 6 servings, providing 668.8 Kj (160 kcal) per 125g serving. The experimental food (bread) was prepared as above with the exception of wheat flour enriched with 18g of barley-derived β-glucan (DKSH Switzerland Ltd., Zurich, Switzerland). Each piece of bread (125g) provided 3g of β-glucan. Both the wheat flour and the barley-derived β-glucan enriched wheat flour were provided by Jotis S.A Food Industry, Athens, Greece.

9.4 Study duration

The duration of the intervention was 3 months.

9.5 Adjustment period

Prior to the commencement of the study participating volunteers were asked to spend a 2 week period of adjustment where they refrained from consuming any type of probiotics or prebiotics.A nutritional assessment was done with a 3-day food diary and their medication was recorded.

9.6 Measurements

Faecal samples were collected at the end of the adjustment period (1st sample), after 1st month (2nd sample), at the end of the 3rd month (3rd sample) and two weeks after the intervention (4th and final sample). This was followed by their microbiological analysis (Fig. 7). Also conducted during the study were somatometric measurements, an assessment of dietary intake via a 3-day food diary, an IPAQ questionnaire that recorded the level of physical activity, a SF-36

questionnaire regarding the volunteer's physical and mental health status, and a questionnaire regarding gastrointestinal symptoms (Fig. 7).

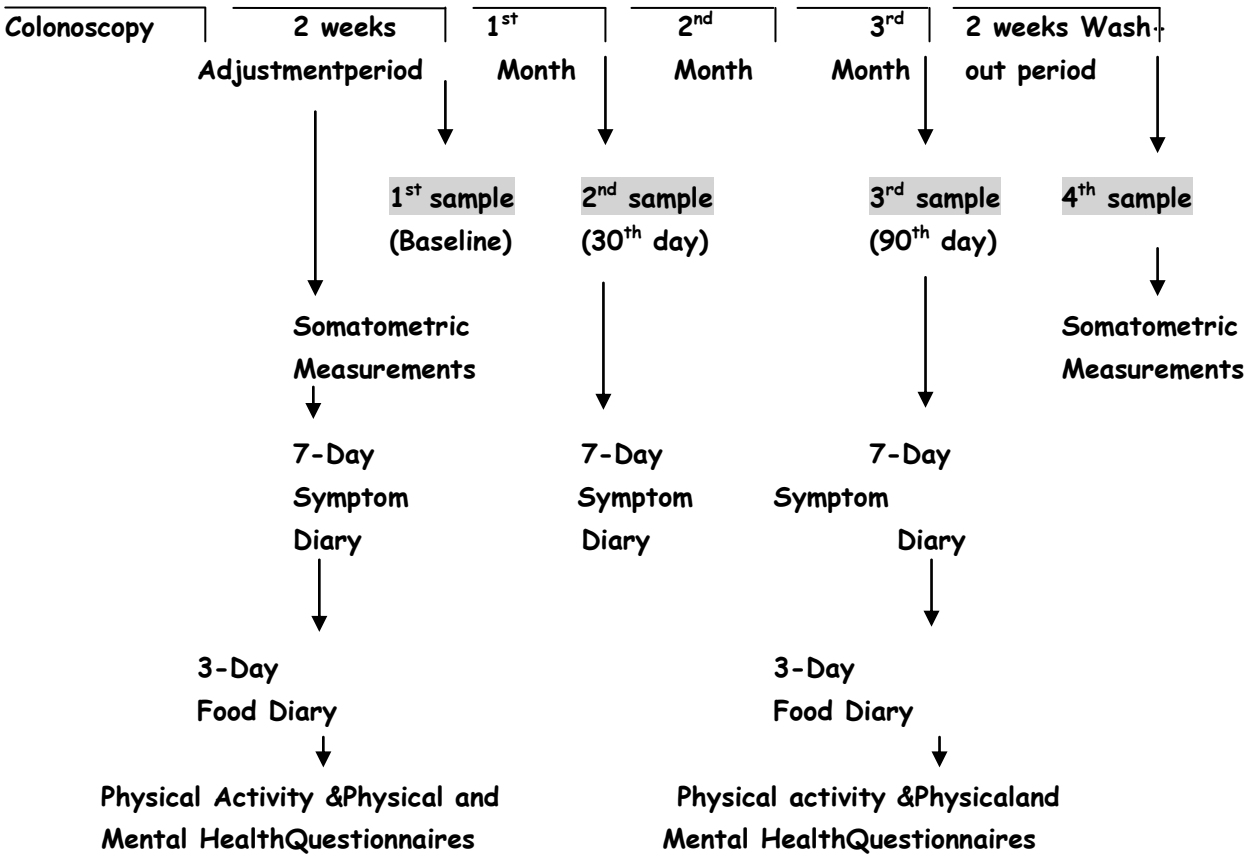


Figure 7.Sample, somatometric measurements, diary and questionnaire timing. Faecal samples were collected at the end of the adjustment period (first sample), after 1st month (2nd sample), at the end of the 3rd month (3rd sample) and the last sample two weeks after the intervention (4th sample).

9.6.1 Enumeration of faecal bacteria

The standard plate count method with different selective and non-selective substrates was used for the enumeration of faecal microorganisms (Fig 8).

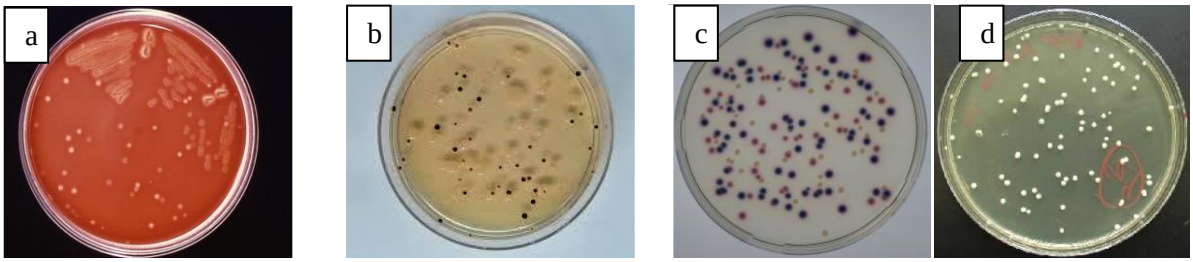


Figure 8.The standard plate count methodwas used for the enumeration of faecal microorganisms such as; (a) *Bacteroides* spp. colonies on Wilkins-Chalgren Agar, (b) *Clostridium* spp. colonies on Tryptose Sulfite Cycloserine (TSC) agar. (c) Total coliforms and *Escherichia coli*on Chromocult agar, and (d) *Lactobacillus* spp. on Rogosa agar.

Subjects were trained to collect faecal specimens from a total passage rapidly into sterile plastic containers. A smaller portion of faeces (~5g) was transferred into a plastic collector with a sterilized spatula and transferred under anaerobic conditions (GTNbag anaer, 45534 Biomurieux-SA, Marcy-l'Etoile, France) to the Laboratory of Microbiology within 1h for microbiological analysis.

Initially, 1g of sample was diluted in 9mL of pre-reduced peptone physiological saline (PPS). The above solution was prepared by dissolving 1g bacteriological peptone (OXOID Basingstoke, Hamshire England) and 8.5g NaCl in 1L of distilled water which was then autoclaved at 121ºC for 15 min [Roy 2001]. After homogenization, serial 10-fold dilutions of the homogenates were performed in PPS under anaerobic environment [5% (v/v) H2: 5% (v/v) CO2: 90% (v/v) N2; BACTRON™ 1.5 Anaerobic Environmental Chamber, SHELLAB, Cornelius, Oregon] (Fig. 9).

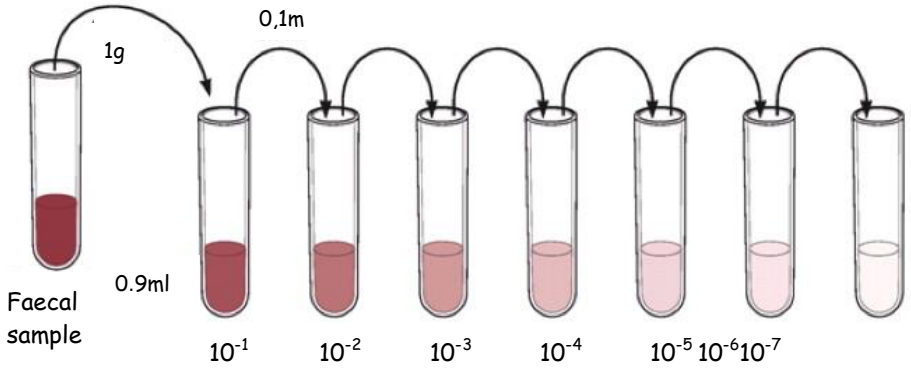


Figure 9.One gram of sample was diluted in 9mL of pre-reduced peptone physiological saline (PPS). After homogenization, serial 10-fold dilutions of the homogenates were performed in PPS under anaerobic environment.

Using these dilutions different selective and non-selective media were inoculated (plating) with 100μL of suspension in order to enumerate different faecal bacterial populations (Table 2). Bacteria were characterized on the basis of colony appearance, Gram's stain, catalase reaction and cell morphology and colony counts were obtained and expressed as a $\log_{10}$ of the colony forming units (CFUs)/g fresh faeces.

Total mesophilic aerobic and anaerobic faecal microbiota. Columbia blood agar was used for the enumeration of the total mesophilic aerobic and anaerobic microbiota [Ellner *et al.* 1966]. This medium is made by dissolving 42g of agar base in 950mL distilled water and by supplementing it with 5g agar (Bacto™ Agar, Difco). It is autoclaved at 121ºC for 15 min. After sterilizing the media the temperature is left to cool until 45-50˚C and then 50mL sterile horse blood (Defidrinated Horse Blood 100mL, REF DIV100, E & C Laboratories Ltd) is added aseptically. After stirring thoroughly, the media is shared in 90mm Petri plates, (about 20mL/dish), in a vertical laminar flow chamber. Petri dishes are inoculated and incubated under aerobic or

anaerobic (BACTRON ™ 1,5 Anaerobic Environmental Chamber, SHELLAB) conditions at 37˚C for 48h.

Table 2.Selected agars and their incubation demands.

Microorganisms	Substrate	Incubation time (h)	Incubation temperature (°C)	Aerobic/ anaerobic conditions
Total mesophilic intestinal microbiota (aerobic & anaerobic)	Columbia Blood Agar (5% defibrinated horse blood)	48	37	Aerobic & Anaerobic
Lactobacillus spp.	Rogosa Agar	48	37	Anaerobic
Bifidobacterium spp.	Beerens Agar	72	37	Anaerobic
Clostridium spp.	Tryptose Sulfite Cycloserine (TSC) Agar	24	37	Anaerobic
Bacteroides spp.	Wilkins-Chalgren Agar supplemented with G-N Anaerobe selective supplement and 5% defibrinated horse blood	48	37	Anaerobic
Enterococcus spp.	Slanetz-Bartley Agar	48	37	Aerobic
Total coliforms & E. *coli*	Chromocult Agar	24	37	Aerobic
Yeasts	Candida Agar	96	35	Aerobic

*Lactobacillus*spp.. The selective medium used for the enumeration and isolation of strains of the genus *Lactobacillus* is Rogosa agar (MERCK) [Rogosa *et al.* 1951]. It is prepared by mixing 74,5g of agar base in 1L of deionized water and the pH is adjusted into 5.5±0.2 at 25˚C with acetic acid (MERCK). It is brought to a boil while stirring constantly (without sterilizing by autoclaving), and then cooled to 40-50˚C before sharing it into Petri dishes. On Rogosa Agar lactobacilli produces round shiny white colonies (Fig. 8 d) and they are countable after 48h of anaerobic incubation at 37°C. Before each count, the presence of different types of colonies was checked microscopically and a representative from each colony type was selected. Microscopic observation after gram-staining and catalase-test was used in order to count all types of lactobacilli, even those that gave non-typical colonies.

*Bifidobacterium*spp.. For counting and isolation of strains of the genus *Bifidobacterium* the selective substrate Beerens' Agar [Beerens 1990, Beerens 1991] was used. This media facilitates the isolation and enumeration of all species of *Bifidobacterium* spp. from any habitat [Beerens 1991] and seems to be the most suitable medium for gut microbiota [Silvi *et al.* 1996]. Media contains 5mL of propionic acid per litre inhibiting the growth of members of *Entrobacteriaceae*

and gram-positive bacteria, as well as strains of *Enterococcus*, *Staphylococcus*, and *Micrococcus* genera. The growth media has 42,5g/L of Columbia agar Base (BBL™, Columbia Agar Base, Becton Dickinson), 5g of agar (Bacto ™ Agar 212750, Difco) with a total final concentration of agar 15g/L, 5g of glucose (Pancreac Quimica SA), 0.5g of cysteine hydrochloride (0.5g/L, MERCK) as a reducing agent, and 5mL propionic acid (99% ACROS) as selective agent of bifidobacteria. At first Columbia Agar Base, agar and cysteine hydrochloride are added in deionized water followed by boiling under continuous stirring. After the medium is cooled to 40-50˚C, glucose and propionic acid are added. pH was adjusted to 5 ± 1 with NaOH 1N solution [Silvi *et al.* 1996]. This media is not autoclaved and it should be used within 48h. The inoculation and the incubation were done anaerobically at 37°C for 72h. The colonies on this media are round, white with irregular margins. Before each count, the presence of different types of colonies was checked microscopically and a representative from each colony type was selected. Microscopic observation after gram-staining and catalase-test was used in order to count all types of bifidobacteria, even those that gave non-typical colonies, and to exclude other anaerobic bacteria which do not exhibit the morphology of bifidobacteria. In some cases, when necessary, endospores-test was used.

*Clostridium*spp..45.6g of Perfringens agar (OPSP) (Oxoid) was dissolved in 1L deionized water after gentle boil [Harmon *et al.* 1971]. Then the solution was sterilized at 121˚C for 10 min. After sterilizing the solution was allowed to cool down to 50˚C and 5mL of filter sterilized antibiotic D-cycloserine solution was added in a concentration of 100 mg/mL. Cycloserine inhibits the growth of the accompanying bacterial biota and the colonies remain smaller. Clostridia colonies are black and easily detectable (Fig. 8 b). This medium is selective mainly for Cl. *perfringens* strains, which seems to be the most common clostridia in the intestine. The recommended anaerobic incubation time at 37ºC is 24h. Before each count, the presence of different types of colonies was checked microscopically and a representative from each colony type was selected. Microscopic observation after gram-staining and catalase-test was used in order to count all types of clostridia, even those that gave non-typical colonies.

*Bacteroides*spp.. Wilkins-Chalgren Agar (Oxoid) was selected for its selectivity due to supplemented antibiotic vancomycin (selective medium for Gram-negative anaerobes) [Wilkins and Chalgren 1976]. This medium is repaired by suspending 43g of Wilkins-Chalgren agar base in 1L of deionized water. It is boiled and sterilized at 121˚C for 15 min. After cooling it to a temperature of 50-55˚C, two vials of G-N Anaerobe Supplement SR0108 (Oxoid) are added aseptically. On this medium *Bacteroides* spp. colonies produce round white/grey colonies (Fig.

8 a). The recommended anaerobic incubation at 37ºC is 48h. Before each count, the presence of different types of colonies was checked microscopically and a representative from each colony type was selected. Microscopic observation after gram-staining and catalase-test was used in order to count all types of bacteroides, even those that gave non-typical colonies.

Enterococcus spp. For the isolation of *Enterococcus* spp. selective Slanetz and Bartley agar (Merck) [Slanetz and Bartley 1957] was chosen. To prepare it, 41.5g of Slanetz and Bartley agar base was dissolved in 1L of distilled water. It is allowed to soak, stirred, and brought to boil in order to dissolve. This media is not autoclaved, but it should be heated for 20 min in order to sterilize. For enterococci the incubation is done aerobically at 35-37°C for 48h. On this medium the enterococci colonies are round with brown colour.

Total coliforms and *Escherichia coli.* Chromocult agar is a highly selected medium for the enumeration of coliforms and *E. coli* recommended by Manafi and Kneifel [Manafi and Kneifel 1989]. For this medium 26.5g of Chromocult agar base is suspended in 1L of distilled water. It is stirred, and brought to boil in order to dissolve. This medium is not to be autoclaved or over heated. Plates are incubated aerobically at 35-37 ° C for 24h. On this medium *E. coli* produces dark blue to violet round colonies and the other faecal coliforms salmon to red round colonies (Fig. 8 c).

Yeasts.For the enumeration of *Candid*a spp. and other yeasts Candida agar was used as recommended by Barr and Collins [Barr and Collins 1966]. To prepare this medium 40g of Candida agar base was suspended in 1L of distilled water. It was stirred well and brought to a boil. This media is not autoclaved. Incubation was done aerobically at 28-35°C for 96h. On this media yeast colonies are usually anywhere from brown to black and with a smooth, pasty appearance.

9.6.2 Short-chain fatty acid concentration (SCFA)

From each sample the following SCFA concentrations were measured: acetate (C2), propionate (C3), iso-butyrate (iC4), butyrate (C4), iso-valerate (iC5), valerate (C5), iso-caproic (iC6), caproic (C6), and heptanoic acid (C7).

The stool sample (1.5g) was homogenized with 4.5mL (1:3) of 0.9% saline and stored at -20°C until analysis. Before analysis samples were homogenized by vortexing and centrifuged at 10,000g for 15 min at 4°C. From the supernatant, 1mL was stored in Eppendorf tubes at -20°C. Eppendorf's were vortexed and centrifuged again at 12,000g for 15min at 4°C. 85µL of the

supernatant was transferred into small GC specific vials. 5µL HCl and 10µL internal standard solution is added carefully, vials are sealed, and stored at -30°C until analysis. Faecal SCFA concentrations were determined by capillary gas chromatography using an Agilent 6890C GC system gas chromatograph (Agilent Technologies) equipped with a 30m x 0.25mm i.d. Nukol column (Supelco, Sigma-Aldrich C., USA), according to Mountzouris *et al.* [Moutzouris *et al.* 2006] (Fig. 10).

Faecal concentrations of individual fatty acids (acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, iso-caproic, caproic, and heptanoic acid) were calculated following appropriate system calibration using a SCFA standard mix (Supelco, Sigma-Aldrich C., USA) and 2-ethyl-butyrate as an internal standard. After measuring iso-butyrate, iso-valerate, and iso-caproate independently, they were expressed as total branched-chain fatty acids (b-SCFA) due to their extremely small concentrations in the samples.Valerate, caproate and heptanoic acid were treated in the same way and presented as other SCFA (o-SCFA). Fatty acids concentrations were expressed as µmol/g (wet weight) faeces and molar ratios (%) were calculated by dividing the amount of each SCFA by the sum of total SCFA multiplied by 100.

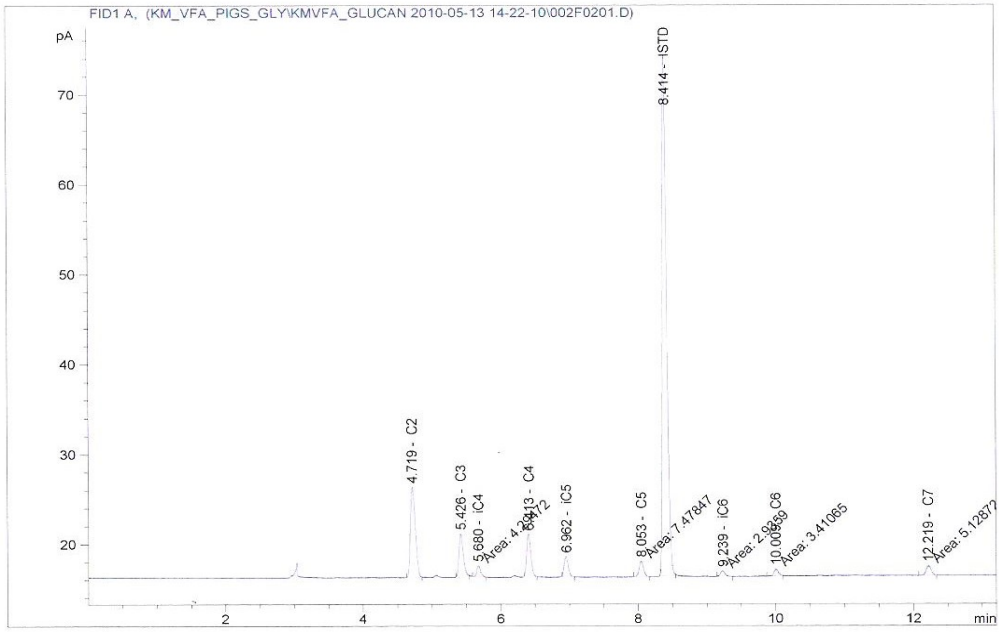


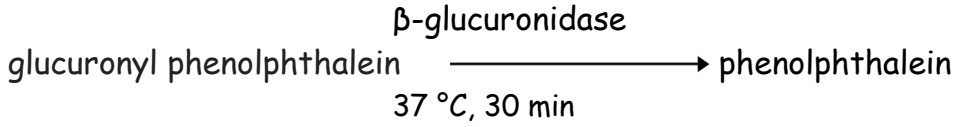
Figure 10.A capillary gas chromatography image.C2, acetate; C3, propionate, C4i, iso-butyrate; C4, butyrate; Ci5, iso-valerate; C5, valerate; iC6, iso-caproic,C6, caproic; and C7, heptanoic acid.

9.6.3 Bacterial enzyme activity

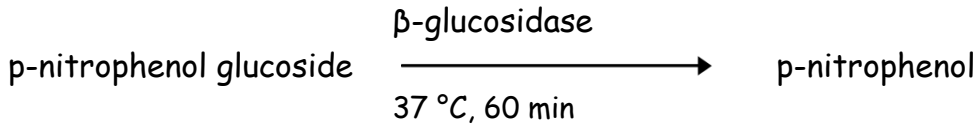
Two faecal microbial enzymes associated with CRC, β-glucuronidase and β-glucosidase, were measured at the baseline, 30th day, 90th day, and two weeks after the trial in order to investigate the influence of β-glucan intake on their activity.

The faecal enzyme activities were determined according to the method of Goldin *et al*. with minor modifications [Goldin *et al.* 1980]. Fresh faecal samples were frozen and kept at -20°C until analysis. After defrosting the faecal samples were suspended in cold PBS to a final concentration of 100 mg/mL. The suspension was sonicated in three 1-min repeats at 4°C in order to rupture the cell walls and then centrifuged at 500g x for 15 min. The supernatant (faecal extract) was collected for the enzyme assays.

β-glucuronidaseassay: 175µL PBS 0.02M (pH 7.0), 25µL EDTA 1mM and 25µL Phenolphthalein-β-glucuronide 10mM were added into a microplate well. The mixture was prewarmed at 37°C for 5 min and then 25µL of the faecal extract was added. The reaction mixture was incubated for 30 min at 37°C. The reaction was terminated by the addition of 50µL of cold glycine buffer. For each sample, a control (faecal extract with reaction mixture without phenolphthalein β-glucuronide substrate, incubated for the same time period) was determined. All samples were run in duplicates. The amount of phenolphthalein released from the enzymatic reaction was quantified by measuring the absorption at 540nm with a Power Wave Microplate Spectrophotometer (BioTek® Instruments, Inc.). The absorbance of control was subtracted from the absorbance of the assay and the concentration of the produced phenolphthalein was calculated from a standard curve of phenolphthalein. The concentration range of standards was from 0.089 to 0.167 (Mean 0.111).



β-glucosidase assay: 175µL PBS 0.02M (pH 7.0), 25µL EDTA 1mM and 25µL p-nitrophenol-D-glucoside 10mM were added into a microplate well. The mixture was prewarmed at 37°C for 5 minand 25µL of the faecal extract was added. The reaction mixture was incubated for 60 min at 37°C. The reaction was terminated by the addition of 50µL of NaOH 1N. For each sample, a control (faecal extract with reaction mixture without p-nitrophenyl β-pyranoside substrate, incubated for the same time period) was determined. All samples were run in duplicates. The amount of p-nitrophenol released from the enzymatic reaction was quantified by measuring the absorption at 450nm with a Power Wave Microplate Spectometer (BioTek® Instruments, Inc.). The absorbance of control was subtracted from the absorbance of the assay and the concentration of the produced p-nitrophenol was calculated from a standard curve of p-nitrophenol. The concentration range of standards was from 0.040 to 0.064 (Mean 0.042).



Total faecal prorein assay: In order to calculate the specific activity of the enzymes, faecal protein concentrations were determined by the modified method of Lowry *et al.* with bovine serum albumin (BSA) as a standard [Lowry *et al.* 1957]. 190µL dH2O, 10µL faecal extract and 50µL Bradfort reagent were added into a microplate hole. The reaction mixture was incubated for 5 min at room temperature. The amount of protein was quantified by measuring the ultraviolet absorption at 595nm with a Power Wave Microplate Spectometer (BioTek® Instruments, Inc.). Concentrations were calculated from a standard curve of bovine serum albumin (BSA) in the range of 0.5-3 µg of BSA. The final enzymatic activities were expressed as nmoles of product per min per milligrams of total faecal protein.

9.6.4 Faecal pH and moisture

One gram of stools was homogenized in 10mL of sterile water using a vortex (Stuart Scientific Autovortex SA6). Then the pH was measured at room temperature using a pH meter (WTW Inolab) and an electrode specially designed for high protein-containing liquids (not destroyed by proteins) (WTW SenTix sp).

The moisture content of faeces was measured by using lyophilisation. Faecal samples were kept stored at -20°C until analysis. Twenty-four hours prior to drying with lyophilisation, the samples were placed at -80°C. Then, 2g of each sample was lyophilized in duplicates for 24h at -54°C, and the remaining dry sample was weighted using an electronical scale.

9.6.5 Faecal water geno- and cytotoxocity

Various components end up in faeces from the colon, such as end-products from microbial activity, and leftovers from food digestion. They can be found dissolved in the faecal water and some of them may have genotoxic or cytotoxic properties. The second part of our study involved testing them in the colon cancer cell line Caco-2 for its genotoxic (ability to induce DNA damage) and cytotoxic (ability to cause cell death) properties.

Faecal water collection:Faecal specimens from a total passage were collected rapidly into sterile plastic containers and transferred to a laboratory for microbiological analysis. Samples were transferred to screw-capped polycarbonated tubes and centrifuged at 18 000rpm for 3h 20 min at

4°C. The supernatant was divided into portions of 1.5mL in Eppendorf containers and stored at –80°C until analysis.

Cell culture:Human small intestinal cells, Caco-2, were thawed and cultured in tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) with 4500mg glucose/Lsupplemented with (10mL/L) penicillin-streptomycin (5000 IU/mL, 5000µg/mL) and foetal bovine serum (100mL/L) (Gibco®, Life Technologies)at 37°C in humidified (95%) atmosphere with 5% CO_2. At ~80% confluence, the cells were subcultivated every week in 1:8 dilutions. The medium was changed on days 2 and 5.

Cell treatment**:** After incubation, cells were harvested, washed twice with PBS, and resuspended by the addition of trypsin (1mL, 0.25% trypsin-EDTA) at 37°C for 5 min and then gently shaken off the plastic flasks. The reaction was terminated by adding DMEM supplemented with faecal bovine serum (100mL/L). To remove the trypsin the cell suspension was transferred to a 15mL Falcon tube, centrifuged (1700 rpm, 5 min), decanted and re-suspended at an appropriate concentration (2 x 10^4 cells/mL) in DMEM.

Cytotoxicity:Cells were incubated with 5% FW for 30min to reach a measurable DNA damage level (in order to avoid cell cytotoxicity exceeding 80%). The cell count and viability was determined by using a Neubauer counter and Trypan Blue dye exclusion assay (50µL + 50µL cell suspension). Cytotoxicity of FW was determined from the equation: % live cells = (unstained cells/stained + unstained cells) x 100.

COMET-assay:Samples were analysed to assess DNA damage using the comet assay to measure the intensity of the comet tail (%). The intensity (TI%) of the comet tail relative to the head reflects the number of DNA breaks in the cell. The samples were treated as previously described [Collins 2004], with minor modifications: the cells were challenged by FW or isotonic saline (9g NaCl/l, pH 7.4, negative control) and incubated (50µL + 950µL cell suspension) for 30 min at 37°C. To stop the incubation, cell suspension was centrifuged (1600 rpm, 5 min, 4°C), the cell pellet was kept on ice, resuspended in ice-cold PBS (1000µL) and centrifuged again for 5 min at the same conditions. The supernatant was discarded and cell pellet was resuspended in ice-cold PBS (250µL) at room temperature. 10µL of the cell suspension was transferred in 130µL of low melting point agarose (0.85% in PBS) and mixed gently by pipetting in 37°C water bath. 70µL of the agarose-cell-suspension was placed toward one end of the precoated slide (1% low melting point agarose in PBS) and a second 70µL drop was placed on the same slide toward the

other end, avoiding the frosted part.As quickly as possible, each drop was covered with a glass coverslip. The slides were placed on a cold glass tray covered with wet Chromatography paper (Whitman™ 3MM) and left in the fridge for 10-15 min. After gently removing the coverlids, the slides were immersed in a lysis buffer (2,5 M NaCl, 100μM EDTA, 10mM TRIS, pH 10) for 2h at 4°C, washed with ddH_2O and then placed in an electrophoresis buffer and allowed to unwind for 40 min. The electrophoresis was run at 25 V (255 mA) for 20 min. Samples were run in duplicates and H_2O_2 was used as a positive control. After the electrophoresis the cells were washed first with a neutralization buffer (0.4 M TRIS, pH 7,5 with HCl) for 10 min at 4°C and then with ddH_2O for 10 min at 4°C. Gels were left to dry overnight. The next day cells were stained with 1mL of SYBR Gold solution (1μL/10mLTE buffer) and left to stain in the dark for 30 min before scoring. Images were analysed at 400x magnification using a fluorescence microscope. From each slide 100 cells were selected and photographed randomly using Comet assay 5.0 (Perceptive Instruments,UK) image analysis software and the percentage of tail DNA was assessed using TriTek Comet ScoreTM Freeware v 1.5 software (Fig. 11). Negative controls (cells without any FW incubation) usually show TI within the range 4-8% and therefore subjects with FW baseline tail intensity <10% were considered to be non-genotoxic and excluded from the study.

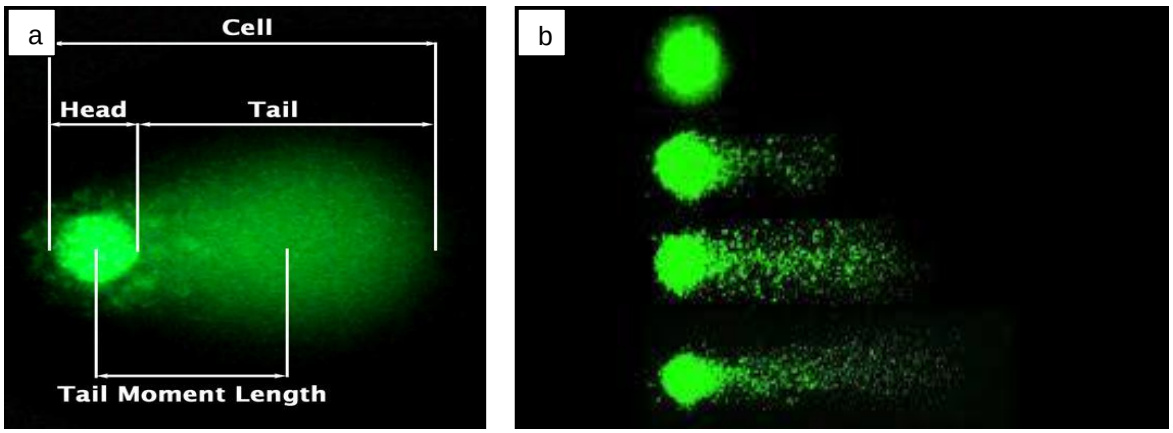


Figure 11.The COMET assay. The COMET assay (single-cell gel electrophoresis) is a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. Cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks (a). The likely basis for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage (b). This can be performed by manual scoring or automatically by imaging software.

9.7 Questionnaires

9.7.1 Gastrointestinal symptoms

Gastrointestinal side effects were evaluated before, during (Day 30-37) and after the intervention (Day 90-97) (Fig. 7) using a 7-day questionnaire in which symptoms (i.e. abdominal pain, bloating, flatulence) were graded from 0 (no symptoms) to 4 (severe symptoms). The 7-d symptom score (sum of symptom intensity during a 7-d period) was graded as 0=no symptoms, 1–7=mild symptoms, 8–14=moderate symptoms and 15–21=severe symptoms. The possible range for each 7-d symptom score estimated to 0–12 and for total symptom score to 0-84, after modification to the method described by Kajander *et al.* [Kajander *et al.* 2005]. Frequency and consistency of evacuations were noted and diarrhoea was defined as one or more watery stools, or more than 3 stools per day. Subjects were also asked to record any appearance of blood in stools (Attachment 1).

9.7.2. Nutrition analysis

Before entering and after finishing the intervention subjects kept a 3-d food diary (Fig. 7). They were told to randomly select three normal week days and record everything they ate or drank. The diaries were then analysed for macro- and micronutrient intake by using Nutritionist Pro™ Diet Analysis software (Axxya Systems LLC.) (Lab. of Cl. Nutrition & Dietetics, Attachment 2).

9.7.3. Physical activity

Before entering and after finishing the intervention subjects filled out an International Physical Activity Questionnaire (IPAQ) - Short Form questionnaire (Fig. 7). IPAQ assessed physical activity undertaken across a comprehensive set of domains including leisure time, domestic and gardening (yard) activities, work-related and transport-related activity. The IPAQ short form asked about three specific types of activity undertaken in the three domains introduced above and sitting. The specific types of activity that were assessed were walking, moderate-intensity activities, and vigorous intensity activities; frequency (measured in days per week) and duration (time per day) were collected separately for each specific type of activity. The items were structured to provide separate scores on walking; moderate-intensity; and vigorous-intensity activity as well as a combined total score to describe the overall level of activity. Total score is the sum of the duration (in minutes) and frequency (days) of walking, moderate-intensity, and vigorous-intensity activity. (www.ipaq.ki.se) (Attachment 3).

9.7.4 Physical and mental health

Before entering and after finishing the intervention subjects filled out a SF-36Health Survey questionnaire (Fig. 7). The SF-36 yields an eight-scale profile of scores as well as a summary of physical and mental measurements The SF-36 is a general report of health status as opposed to others more specific to age, disease, or treatment group [Ware and Gandek 1998]. (Attachment 4).

9.8 Statistics

Bacterial counts (log_{10} CFU/g faeces), SCFA concentration, enzyme activity, pH, dry-weight Tail intensity, gastrointestinal symptoms, physical activity, physical and mental health, and macro- and micronutrient intake at each sampling time respectively were compared using Repeated Measures ANOVA (RM-ANOVA) for parametric and the Friedman test for non-parametric data. Intragroup analyses were performed respectively using paired-sample t-test for parametric data and the Wilcoxon signed ranks test for non-parametric data. The statistical analysis of the results was performed by the software program SPSS® for Windows Release 13.0 and the significance threshold was set at 5% ($P<0.05$).

9.9 Ethics

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Bioethics Committee of Harokopio University (20/29-05-2008). Written consent forms were obtained from all subjects. This study is registered in www.ClinicalTrials.gov.

10. Results

10.1 Volunteers' characteristics

From 69 polypectomised men and women (mean age 61.1 years) recruited into the study, only 57 completed (Table 3). The main dropout reason was the consumption of antibiotics during the study. From 57 volunteers according to the randomized design of the study 27 volunteers (16 men and 11 women) consumed the placebo and 30 volunteers (13 men and 17 women) the experimental bread. Sufficient volume of FW was collected from 54 patients. No significant differences were detected in the age, gender, BMI, smoking habits, physical and mental health score or pathology of the adenomas between the two groups. The controls were found to be physically more active than subjects in the β-glucan group both before entering and at the end of the intervention (check chapter: 10.8 Physical activity).

Table 3. Selected characteristics of volunteers.

	Controls	β-glucan	*p*-value
Age	59.8 ± 7.3	62.4 ± 8.7	0.767
Men/Women *(n)*	16/11	13/17	0.230
BMI	29.8 ± 4.6	27.8 ± 3.7	0.072
Smoking %	44	36	0.550
Physical activity (kcal/d)	3011.9 ± 957.6	2463.7 ± 477.4	0.005
Physical health score	49.34 ± 8.48	49.05 ± 6.92	0.788
Mental health score	45.28 ± 10.43	45.73 ± 9.75	0.815
Pathology (*n*)			
Hyperplastic polyps	4	3	0.580
Serrated adenoma	4	3	0.580
Tubular adenoma	8	8	0.804
Tubulovillus adenoma	9	11	0.792
Tubulovillus adenomas with high-degree dysplasia	2	5	0.288

10.2 Faecal microbiota

The counts of selected faecal microbial populations are presented in Table 4.

10.2.1 Total mesophilic anaerobic or aerobic bacteria

Total aerobic bacteria were measured at 8.43 $\log_{10}$ CFU/g for the β-glucan group and at 8.56 $\log_{10}$ CFU/g for the control group, while the anaerobes reached 9.78 $\log_{10}$ CFU/g for the β-glucan and 9.72 $\log_{10}$ CFU/g for the control group at the baseline. During the intervention we did not observe any significant changes in total mesophilic anaerobic or aerobic bacteria between the two study groups (Fig.12, Table 4).

Table 4. Faecal bacterial counts (log_{10}CFU/g faeces) in the control group (n=27) and in the β-glucan (n=30) group at the baseline, Day 30, Day 90, and 2 weeks after the intervention.

	Baseline		Day 30		Day 90		2 weeks after the intervention		
Bacterial group	**Control**	**β-glucan**	**Control**	**β-glucan**	**Control**	**β-glucan**	**Control**	**β-glucan**	***p*-group**
Total aerobes	8.56 ± 0.84	8.43 ± 0.80	8.30 ± 0.88	8.60 ± 0.66	8.38 ± 0.78	8.46 ± 0.88	8.43 ± 0.74	8.43 ± 1.20	0.742
Total coliforms	7.53 ± 1.02	7.49 ± 0.93	7.11 ± 1.11	7.28 ± 1.07	7.00 ± 1.01	7.37 ± 0.95	7.34 ± 0.85	7.25 ± 1.31	0.633
E. coli	6.74 ± 2.05	7.08 ± 1.00	6.84 ± 1.63	7.22 ± 1.08	6.85 ± 1.16	7.27 ± 0.95	7.23 ± 0.92	7.25 ± 1.30	0.253
Enterococcus spp.	6.89 ± 1.46	6.57 ± 1.38	6.31 ± 1.49	6.65 ± 1.41	6.03 ± 1.59[c]	6.55 ± 1.46	6.12 ± 1.52[c]	6.12 ± 1.46[d]	0.674
Candida spp.[a]	3.13 ± 1.22	2.82 ± 1.59	3.01 ± 1.44	2.90 ± 1.23	3.03 ± 1.63	2.80 ± 1.34	3.17 ± 1.31	2.81 ± 1.26	0.403
Total anaerobes	9.72 ± 0.52	9.78 ± 0.57	9.59 ± 0.70	9.84 ± 0.52	9.85 ± 0.64	9.87 ± 0.64	9.67 ± 0.56	9.72 ± 0.65	0.441
Bacteroides spp.	8.99 ± 1.00	8.95 ± 0.67	8.80 ± 1.08	9.03 ± 0.85	9.20 ± 0.99	8.87 ± 1.19	9.07 ± 0.88	8.76 ± 1.13	0.589
Lactobacillus spp.	6.20 ± 1.60	6.41 ± 1.52	6.18 ± 1.92	6.31 ± 1.64	6.18 ± 1.47	6.63 ± 1.52	6.15 ± 1.41	6.65 ± 1.56	0.404
Clostridium perfringens	4.87 ± 1.54	4.66 ± 1.24	4.30 ± 1.21	4.85 ± 1.10	4.80 ± 1.47	4.58 ± 1.26	5.27 ± 1.27	4.93 ± 1.43	0.857
Bifidobacterium spp.[b]	8.12 ± 1.46	8.37 ± 2.00	8.15 ± 1.48	8.60 ± 1.71	8.89 ± 0.78[d,e]	8.80 ± 1.30[c]	8.72 ± 0.97	8.43 ± 2.21	0.287

All values are *x* ± S.D.; CFU, colony forming units.

[a]*Candida*spp., control group n=24, β-glucan group n=28; [b]*Bifidobacterium* spp., control group n=25, β-glucan group n=28.

[c]Significantly different from the baseline; [d]Significantly different from the Day 90; [e]Significantly different from the Day 30 (*p*<0.05).

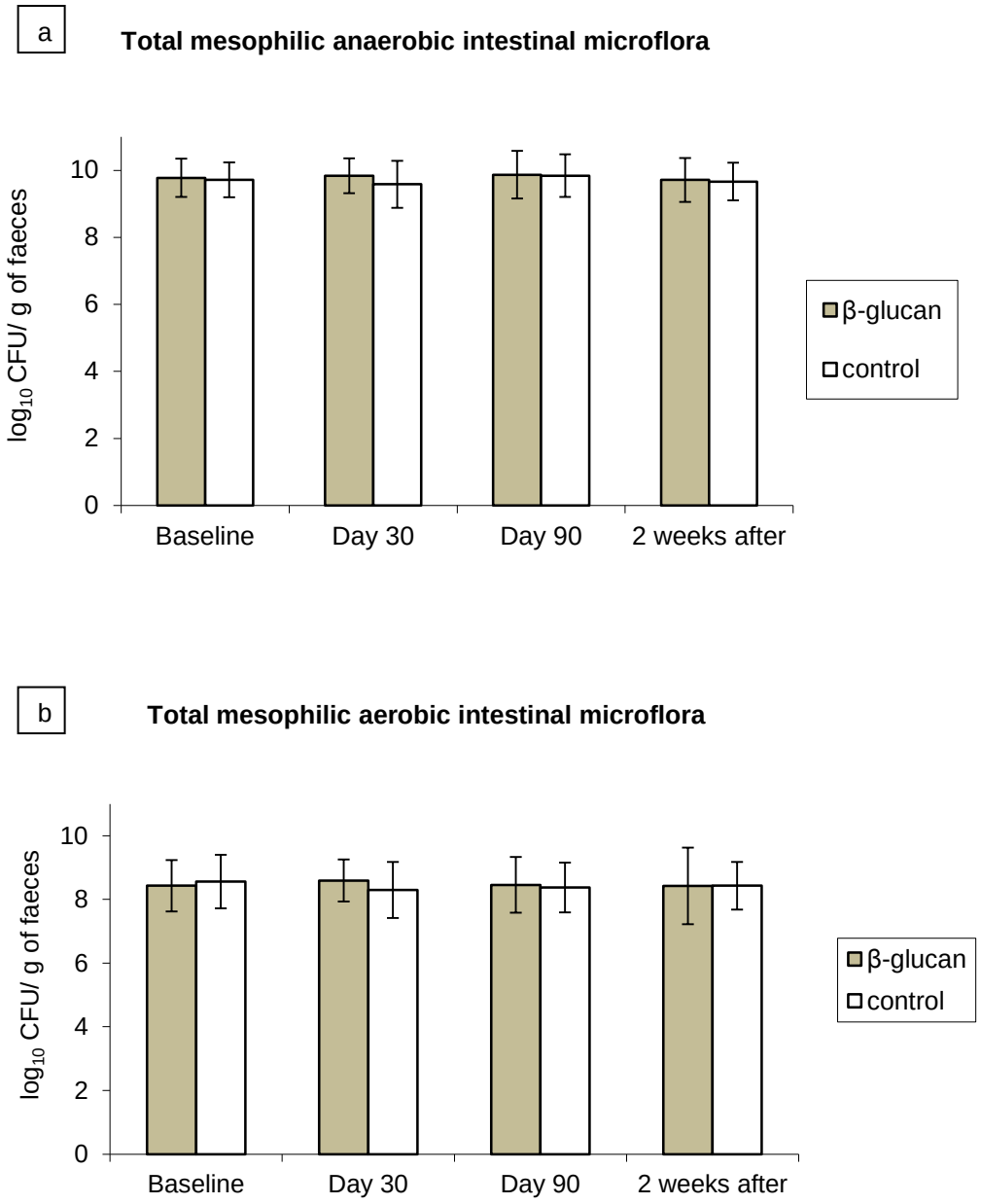


Figure 12. Total mesophilic anaerobic (a) and aerobic (b) faecal bacteria in the control group (n=27) and in the β-glucan group (n=30) during the intervention.

10.2.2 Total coliforms and *Escherichia coli*

The total coliforms and *E.coli* counts measured in the β-glucan group did not change during the trial compared to control or baseline. The mean value at the baseline for the total coliforms was 7.49 $\log_{10}$ CFU/g for the β-glucan group and 7.53 $\log_{10}$ CFU/g for the control group. *E.coli* was measured at 7.08 $\log_{10}$ CFU/g in the β-glucan group and at 6.74 $\log_{10}$ CFU/g in the control group at the baseline (Fig.13, Table 4).

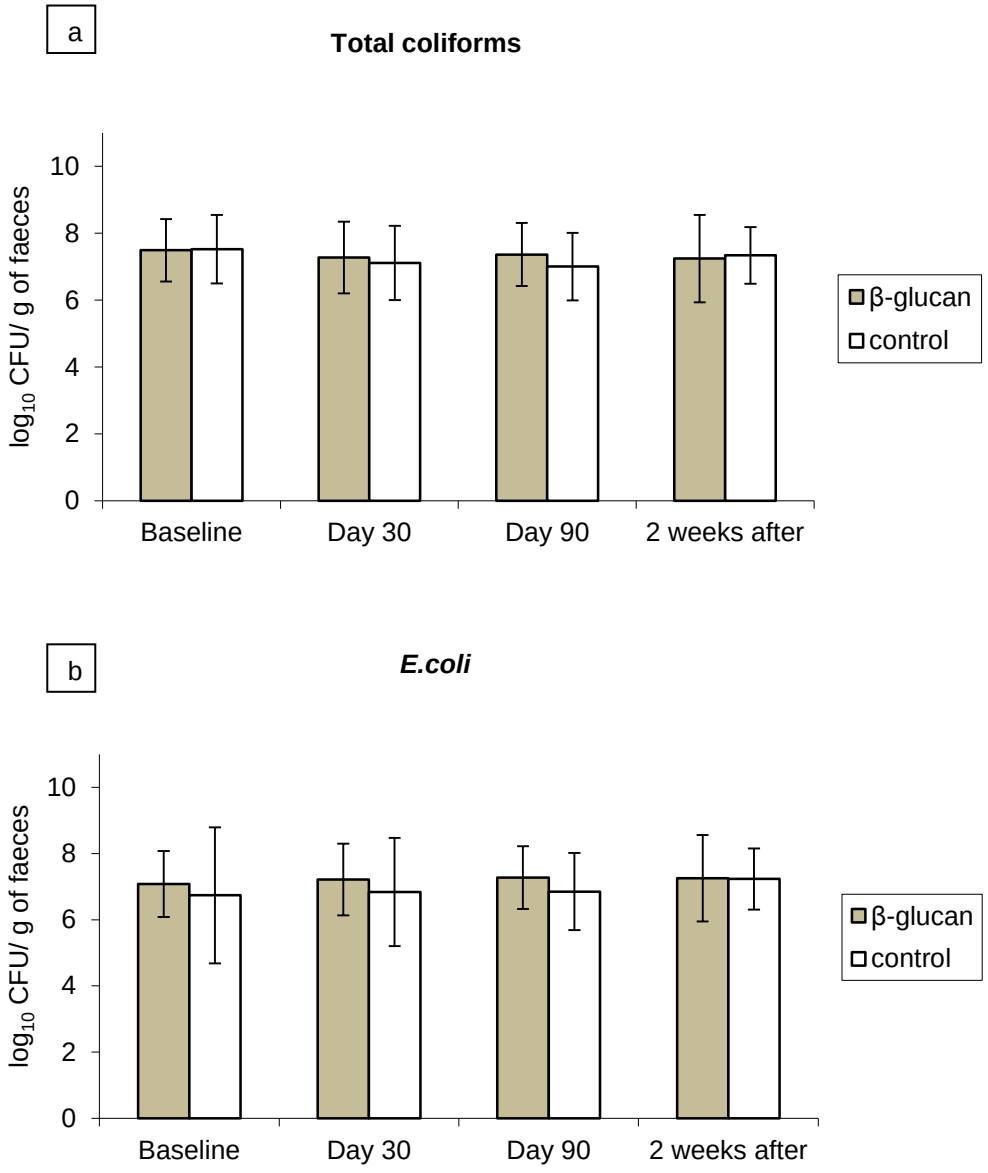


Figure 13. Total coliforms (a) and *E.coli* (b) counts in the control group (n=27) and in the β-glucan group (n=30) during the intervention.

10.2.3 *Enterococcus* spp.

The baseline *Enterococcus* spp. counts were 6.57 $\log_{10}$ CFU/g for the β-glucan group and 6.89 $\log_{10}$ CFU/g for the control group. We did not observe any significant changes in the enterococcal levels between the two study groups. Enterococci were decreased in the β-glucan group 2 weeks after the intervention compared to the 90th day (p=0.037), and in the control group on the 90th day (p=0.010) and 2 weeks after the intervention compared to the baseline (p=0.014) (Fig. 14, Table 4).

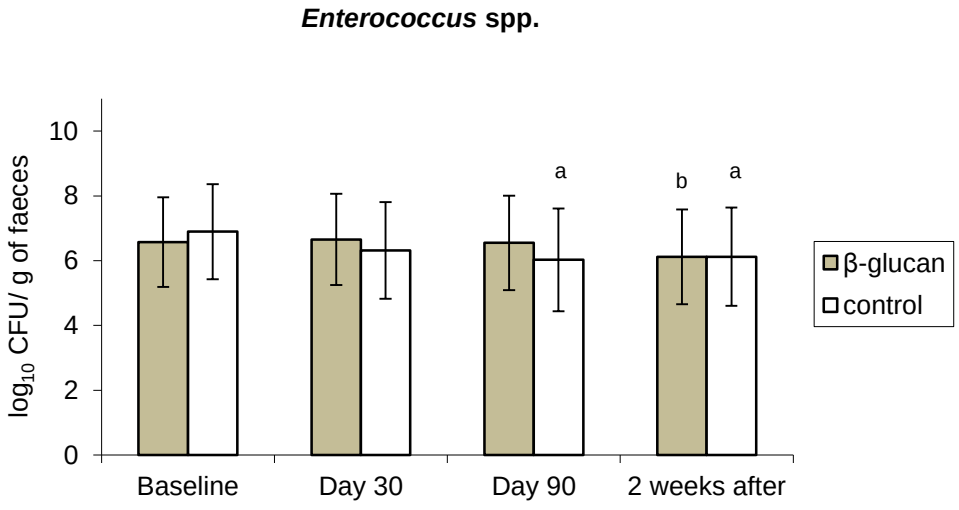


Figure 14.*Entrococcus* spp.counts in the control group (n=27) and in the β-glucan group (n=30) during the intervention. [a]Significantly different from the baseline, (p<0.05); [b]Significantly different from the Day 90 (p<0.05).

10.2.4 *Bacteroides* spp.

Bacteroides spp. were measured at 8.95 $\log_{10}$ CFU/g in the β-glucan group at the baseline and did not change during the intervention or compared to the control group (Fig. 15, Table 4).

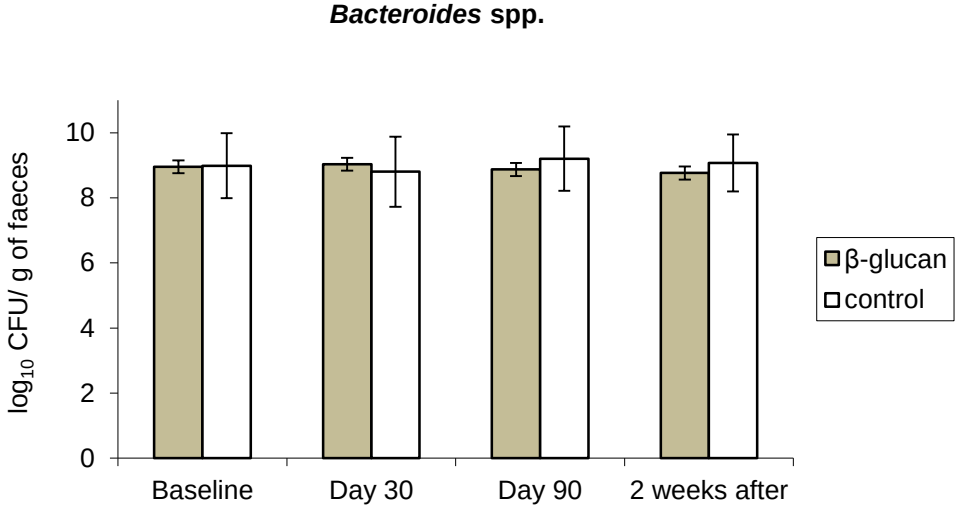


Figure 15.*Bacteroide*s spp. counts in the control group (n=27) and in the β-glucan group (n=30) during the intervention.

10.2.5 *Clostridium perfringens*

Clostridium perfringens counts did not change significantly between the two study groups. In the β-glucan group *Clostridium perfringens* was measured at the highest level, 4.85 $\log_{10}$

CFU/g, on the 30th day of the intervention, while in the control group clostridia reached 5.27 $\log_{10}$ CFU/g on the 90th day. A close to significant difference between the two study groups was observed on 30th day of the intervention (p=0.074). In detail, an increase in clostridial levels tended to be more frequently observed in subjects consuming β-glucan compared to the control group on 30th day of the intervention (53.3% vs. 29.6%, p=0.070), with the average change in initial clostridia counts on the 30th day of the intervention being significantly higher in the β-glucan group compared to the control group (0.17 ± 1.20 vs. -0.57± 1.47, p=0.042) (Fig. 16, Table 4).

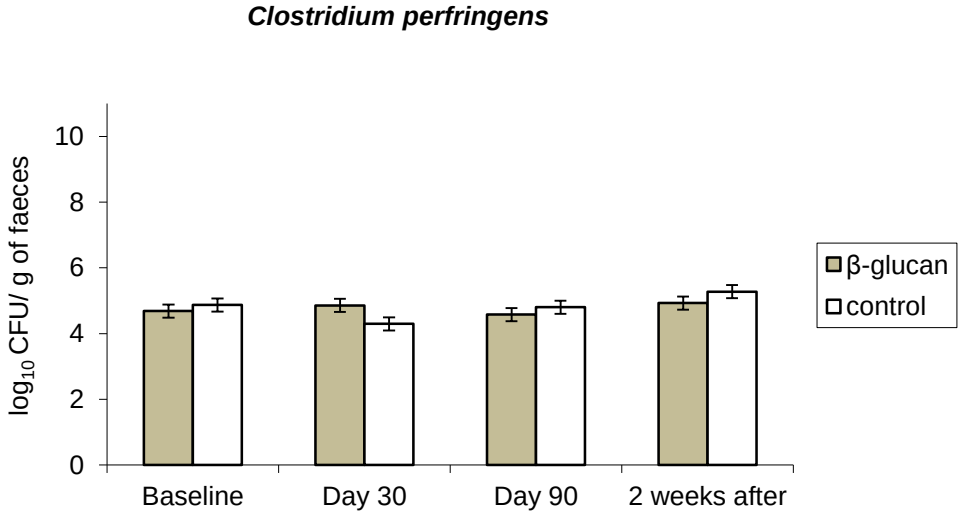


Figure 16.*Clostridium perfringens* counts in the control group and in the β-glucan group during the intervention.

10.2.6 *Bifidobacterium* spp.

No significant changes were recorded in bifidobacteria counts between the two study groups. Levels of bifidobacteria had a trend to increase during the intervention in both study groups. Group specific analysis for bifidobacteria in the β-glucan group showed a significant increase on the 90th day (8.80 $\log_{10}$ CFU/g) compared to the baseline (8.29 $\log_{10}$ CFU/g) (p=0.011); however bifidobacteria was also found to be increased in the control group on the 90th day (8.91 $\log_{10}$ CFU/g) compared to the baseline (8.12 $\log_{10}$ CFU/g) (p=0.019) and to 30th day (8.15$\log_{10}$ CFU/g) (p=0.030) (Fig. 17, Table 4).

Two subjects in both the β-glucan group and in the control group had no measurable bifidobacterial levels in their baseline sample and were therefore excluded from the statistical analysis. The two subjects from the β-glucan group reached detectable bifido counts from the 30th day of the intervention, although 2 weeks after the trial one of them had yet again no

detectable levels of*Bifidobacterium* spp. The two subjects in the control group evolved subsequently: one subject had detectable bifido counts 2 weeks after the intervention and the other subject remained throughout the study with no detectable bifidobacteria counts.

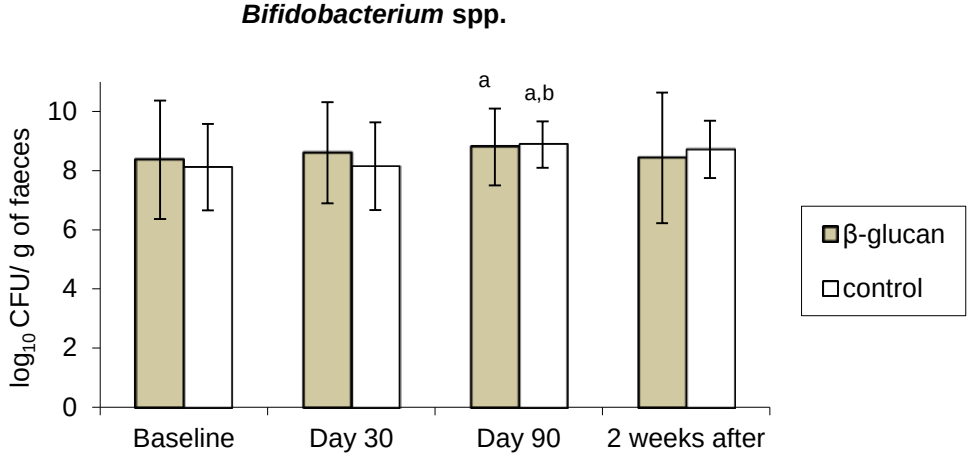


Figure 17.*Bifidobacterium* spp. in the control group (n=25) and in the β-glucan group (n=28) during the intervention. [a]Significantly different from the baseline, (p<0.05); [b]Significantly different from Day 30 (p<0.05).

10.2.7 *Lactobacillus* spp.

In the β-glucan group *Lactobacillus* spp. counts were measured at the highest value on the 90th day at 6.63 $\log_{10}$ CFU/g and in the control group they practically remained at the same level (6.20 $\log_{10}$ CFU/g at the baseline). During the intervention we did not observe any significant changes in lactobacilli count between the two study groups. In the β-glucan group *Lactobacillus* spp. counts had a trend to increase on the 90th day (6.63 $\log_{10}$ CFU/g) compared to the 30th day (6.31$\log_{10}$ CFU/g) without reaching significance (p=0.088) (Fig. 18, Table 4).

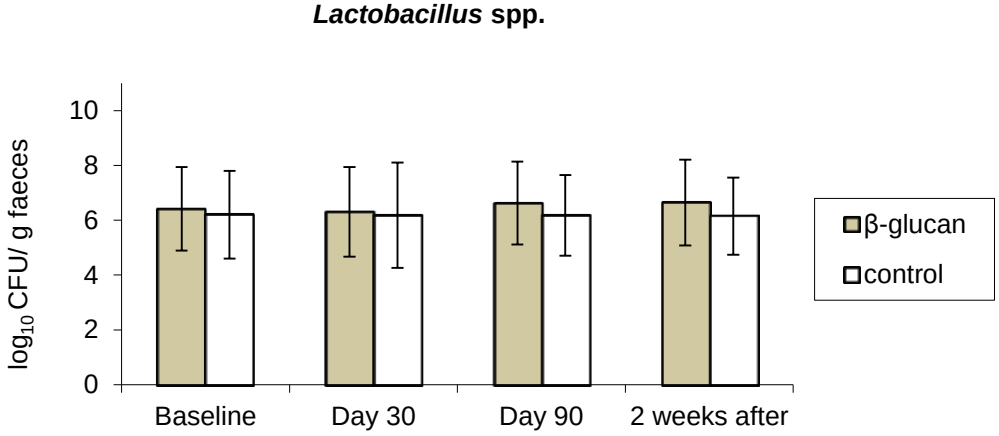


Figure 18.*Lactobacillus* spp.in the control group (n=27) and in the β-glucan group (n=30) during the intervention.

10.2.8 *Candida* spp.

The *Candida* spp. levels were at 3.01 $\log_{10}$ CFU/g in the control group and at 2.89 $\log_{10}$ CFU/g for the β-glucan group at the baseline. No changes in *Candida* spp. counts were recorded during the intervention compared to the control (Fig. 19, Table 4).

Two subjects in the β-glucan and three subjects in the control group had no measurable *Candida* spp. levels in their baseline sample and were therefore excluded from the statistical analysis. The two subjects from the β-glucan group reached detectable *Candida* spp. counts from the 30th day of the intervention, although the samples taken on the 90th day and 2 weeks after the trial for both of them again showed no detectable levels of *Candida* spp. The three subjects in the control group evolved subsequently: one subject had low level, detectable *Candida* spp. counts from the 30th day while the other two subjects remained throughout the study with no detectable *Candida* spp. counts.

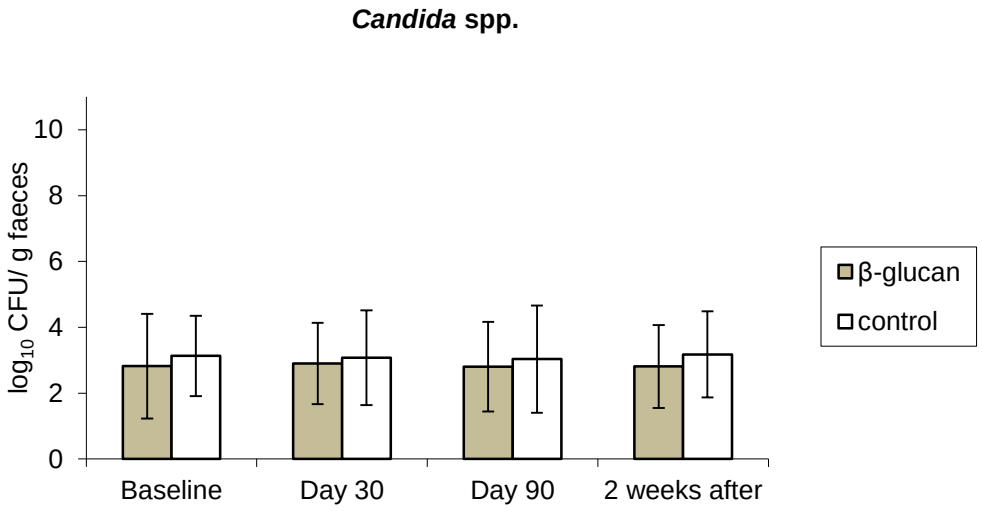


Figure 19.*Candida*spp.in the control group (n=24) and in the β-glucan group (n=28) during the intervention.

10.3 Short-chain fatty acid (SCFA) concentration

Acetate, propionate, and butyrate concentrations, expressed as molar ratios,were measured in all samples and are presented in Figure 20. We did not record any changes in acetate or propionate molar ratio during the intervention. Butyrate molar ratio had a significant difference between the two study groups on the 90th day of the intervention (p=0.007). In detail, an increase in butyrate molar ratio tended to be more frequently observed in controls compared to subjects consuming β-glucan on the 90th day of the intervention (59.3% vs. 50.0%, p=0.050), with an average change in initial butyrate molar ratio on the 90th day of the

intervention being higher in the control group compared to the β-glucan group (2.49 ± 6.51 vs. -1.34 ± 7.88, p=0.483) (Table 5).

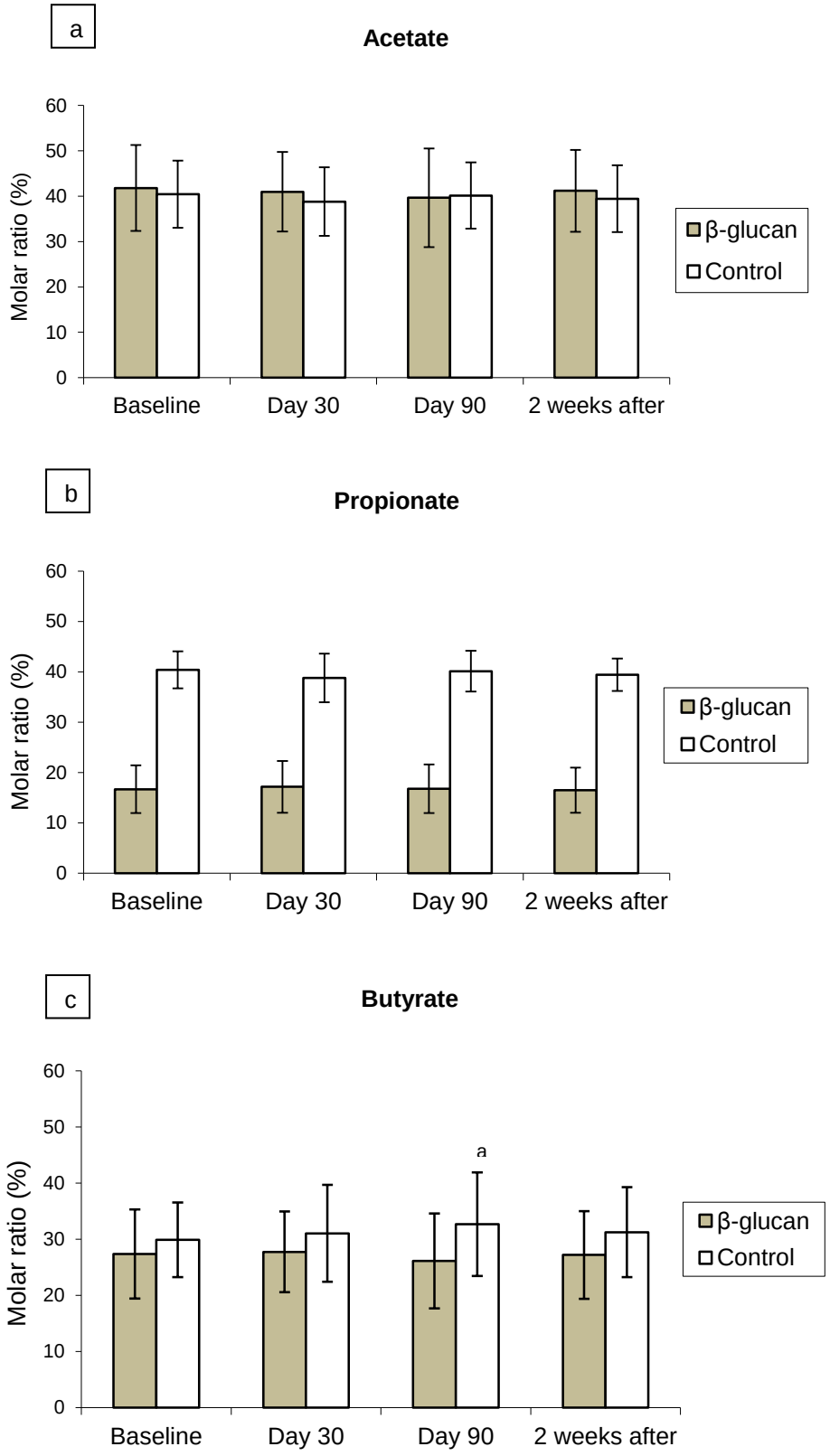


Figure 20. Acetate (a), propionate (b) and butyrate (c) molar ratios in the control group (n=27) and in the β-glucan group (n=30) during the intervention. [a]Significantly different from theβ-glucan group (p>0.05).

The molar ratio of other SCFA (o-SCFAs, valerate, caproate, and heptanoic acid) was significantly increased on the 90th day in the β-glucan group, compared to the control group (p=0.033) (Fig. 21 a). This was most likely due to the significant increase of valerate molar ratio (p=0.024)(Fig. 21 b). The branched-chain fatty acids (b-SCFAs, iso-butyrate, iso-valerate, and iso-caproate) molar ratio for the glucan group reached its highest value on the 90th day of the intervention, but did not exhibit significant difference compared to the control group (p=0.059). Simultaneously, iso-valerate molar ratio was significantly higher in the β-glucan group compared to the control group (p=0.026) (Table 5).

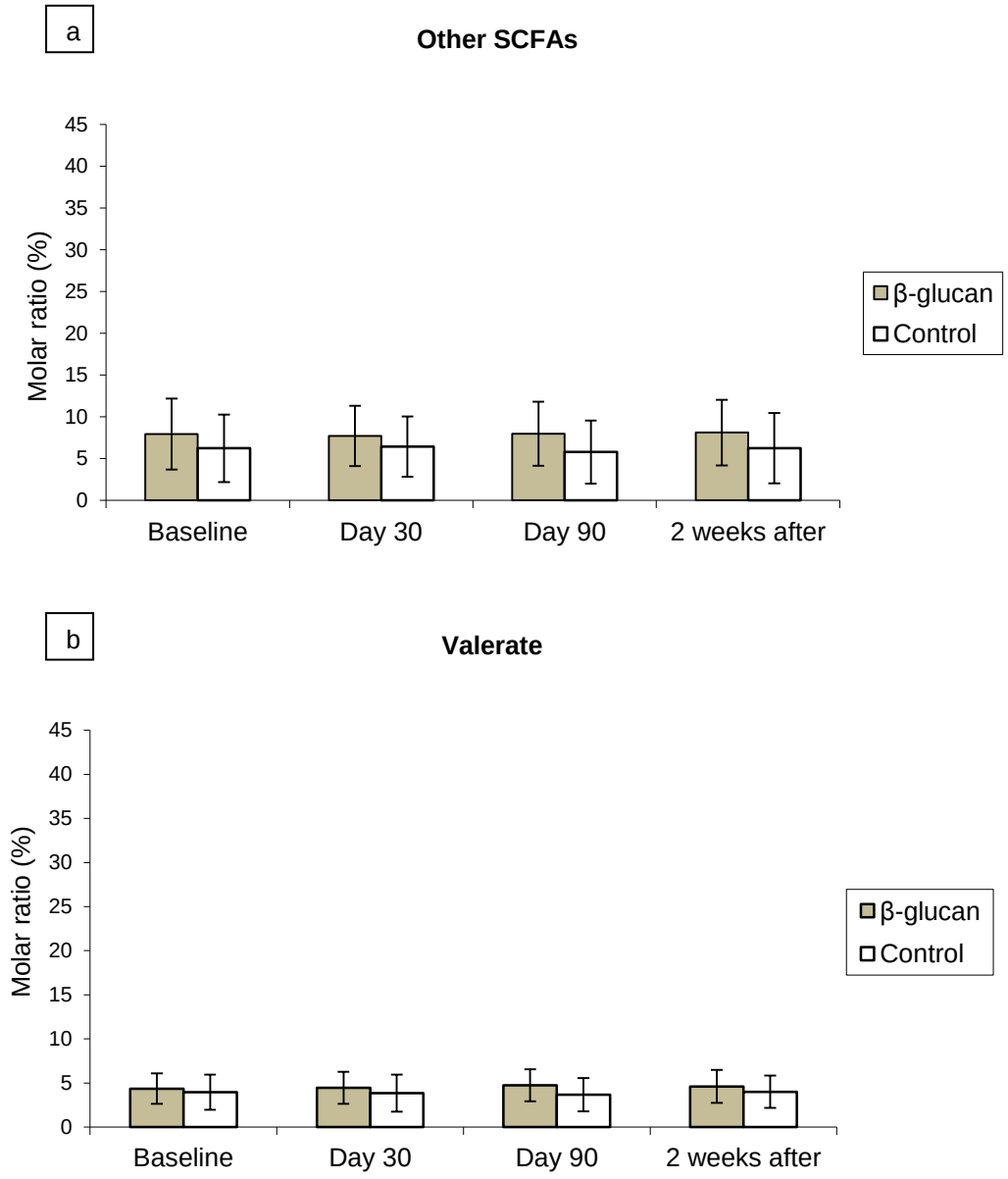


Figure 21. (a) The sum of other SCFAs molar ratio (valerate, caproate and heptanoic acid) and (b) the valerate molar ratio in the control group (n=27) and in the β-glucan group (n=30) during the intervention.
[a]Significantly different from the control (p<0.05).

Table 5. Faecal SCFA concentrations (molar ratio %) in the control group (n=27) and in the β-glucan group (n=30) at the baseline, Day 30, Day 90, and 2 weeks after the intervention.

	Baseline		Day 30		Day 90		2 weeks after the intervention		
	Control	β-glucan	Control	β-glucan	Control	β-glucan	Control	β-glucan	*p*-group
Acetate	40.42 ± 7.39	41.81 ± 9.47	38.80 ± 7.56	40.97 ± 8.78	40.14 ± 7.30	39.65 ± 10.89	39.44 ± 7.35	41.19 ± 9.01	0.549
Propionate	17.18 ± 3.68	16.71 ± 4.75	17.32 ± 4.81	17.17 ± 5.15	15.99 ± 4.04	16.81 ± 4.82	15.97 ± 3.20	16.51 ± 4.50	0.859
Butyrate	29.91 ± 6.65	27.38 ± 7.94	31.04 ± 8.64	27.72 ± 7.19	32.68 ± 9.22[a]	26.12 ± 8.43	31.24 ± 8.02	27.18 ± 7.83	0.023[c]
i-butyrate[d]	2.26 ± 1.80	1.83 ± 0.98	1.88 ± 1.48	1.79 ± 0.92	1.65 ± 1.31	2.12 ± 1.08	1.84 ± 1.69	2.18 ± 1.83	0.821
Valerate	3.94 ± 1.99	4.36 ± 1.73	4.12 ± 2.11	4.45 ± 1.81	3.60 ± 1.88	4.74 ± 1.80[b]	3.86 ± 1.85	4.60 ± 1.87	0.146
i-valerate	3.95 ± 2.78	3.87 ± 2.29	3.84 ± 3.09	4.35 ± 2.66	3.67 ± 3.35	5.22 ± 3.03[b]	4.00 ± 3.56	4.60 ± 3.34	0.327
Caproate	1.79 ± 1.93	2.70 ± 2.33	1.77 ± 1.53	2.53 ± 2.04	1.64 ± 1.82	2.54 ± 2.01	1.90 ± 2.16	2.85 ± 2.26	0.061
i-caproate[e]	0.24 ± 0.12	0.86 ± 1.58	0.33 ± 0.33	0.52 ± 0.61	0.32 ± 0.29	0.42 ± 0.38	0.25 ± 0.31	0.35 ± 0.21	0.154
Heptanoate[f]	0.61 ± 0.47	0.97 ± 1.30	0.69 ± 0.76	0.81 ± 0.80	0.72 ± 0.66	0.79 ± 0.65	0.64 ± 0.84	0.70 ± 0.55	0.147
b-SCFA	6.27 ± 4.34	6.17 ± 3.63	5.82 ± 4.46	6.43 ± 3.36	5.42 ± 4.53	7.58 ± 3.94	6.03 ± 5.12	7.01 ± 4.84	0.330
o-SCFA	6.23 ± 4.03	7.94 ± 4.25	6.43 ± 3.60	7.71 ± 3.62	5.78 ± 3.78	7.99 ± 3.83[b]	6.26 ± 4.21	8.11 ± 3.93	0.057

All values are x ± S.D.

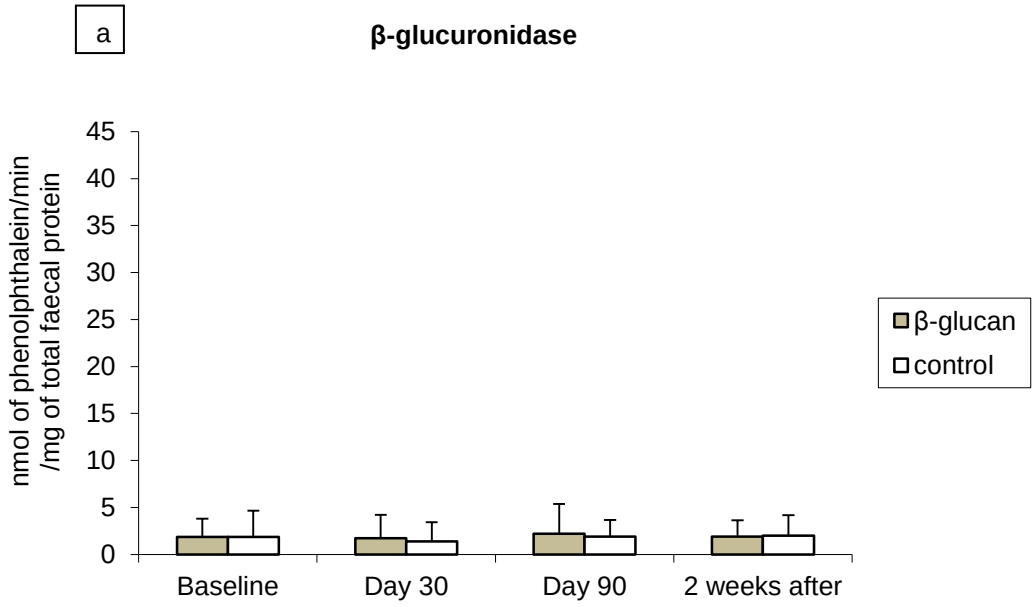
[a]signifantly different from the β-glucan group; [b]significantly different from the control group;
[c]*p*>0.05.
[d]i-butyrate, control group n=26, β-glucan group n=30; [e]i-caproate, control group n=9, β-glucan group n=16; [f]Heptanoate, control group n=20, β-glucan group n=27.

b-SCFA, sum of branched chain short-chain fatty acids; o-SCFA, sum of other short-chain fatty acids.

10.4 Bacterial enzyme activity

The activity of the two faecal bacterial enzymes, β-glucuronidase and β-glucosidase, is presented in Figure 22. The activity of β-glucuronidase was expressed in nmol of phenolphthalein/min/mg of total faecal protein and its mean baseline value was 1.86 nmol/ min/mg for the β-glucan group and 1.87 nmol/min/mg for the control group, while the activity of β-glucosidase was expressed in nmol of p-nitrophenyl/min/mg of faecal protein and its mean baseline value was 23.27 nmol/min/mg for the β-glucan group and 13.28 nmol/min/mg for the control group. We did not record any significant changes in the activity of these two faecal bacterial enzymes during this intervention (Fig. 22 a,b).

Six subjects in the β-glucan group and seven subjects in the control group had no measurable β-glucuronidase levels in their baseline sample and were therefore excluded from the statistical analysis. All of them remained throughout the study with no detectable β-glucuronidase levels. One subject in the control group had no measurable β-glucosidase levels in her baseline sample and therefore was excluded from the statistical analysis. This subject reached low detectable β-glucosidase levels on the 90th day of the intervention, which further increased 2 weeks after the trial.



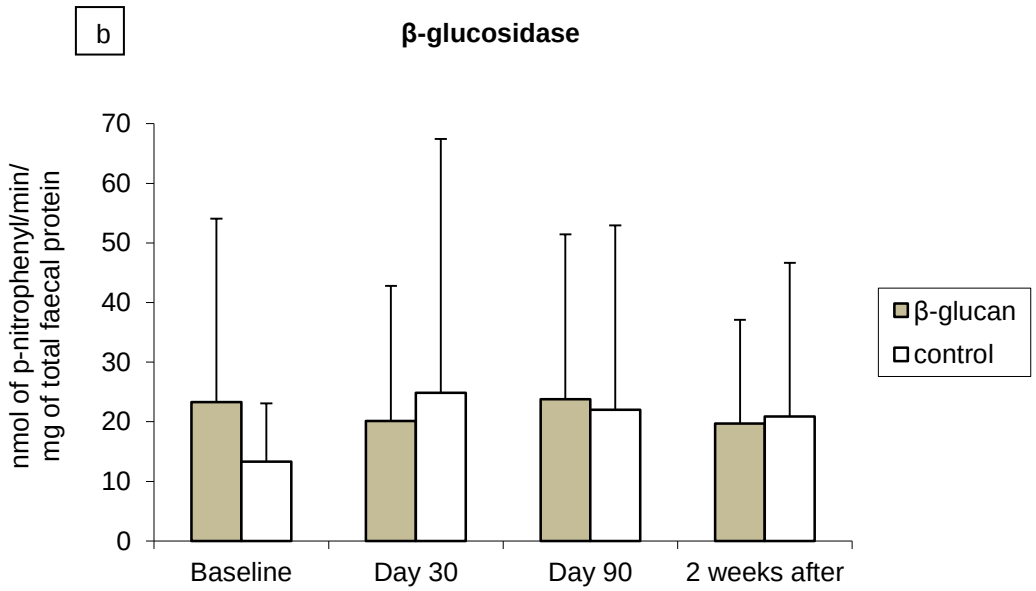
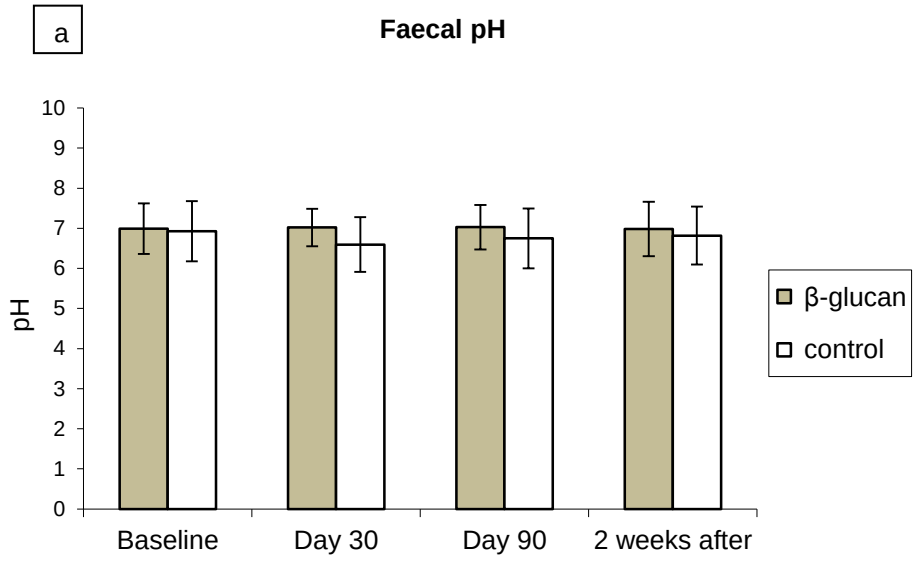


Figure 22. (a) Bacterial enzyme activity of β-glucuronidase expressed in nmol of phenolphthalein/min/mg of total faecal protein in the control group (n=20) and in the β-glucan group (n=24), and (b) bacterial enzyme activity of β-glucosidase expressed in nmol of p-nitrophenyl/min/mg of total faecal protein in the control group (n=26) and in theβ-glucan group (n=30) during the intervention.

10.5 Faecal pH and moisture

The subjects mean faecal pH at baseline was 6.96. During the intervention we observed a minor decrease in faecal pH on the 30th day in the control group, resulting in a significantly lower value compared to the β-glucan group (from 6.93 to 6.59, p=0.008). On the 90th day the pH value increased again (Fig. 23 a).

The subjects mean baseline faecal moisture was 65.8%. We did not record any significant changes in faecal moisture during this intervention (Fig. 23 b).



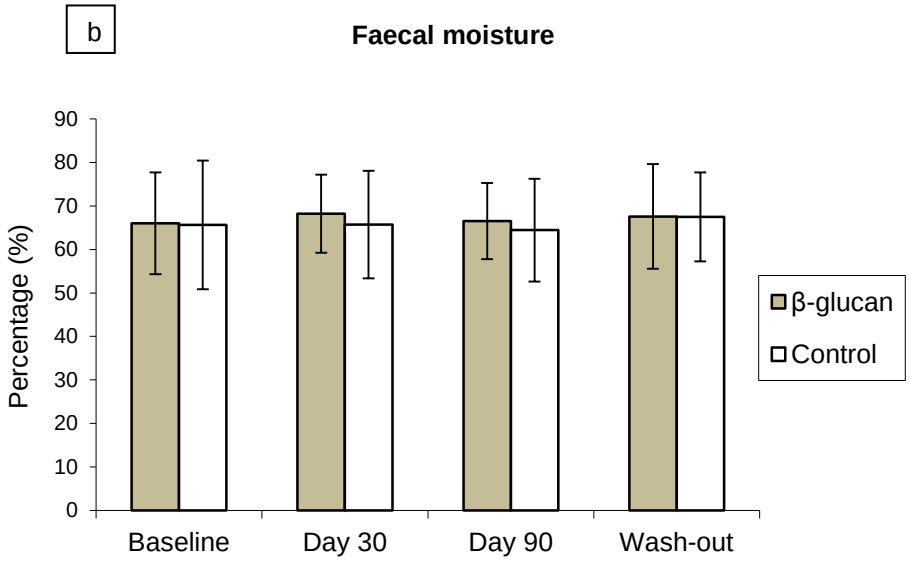


Figure 23. (a) Faecal pH in the control group (n=27) and in the β-glucan group (n=30) during the intervention. (b) Faecal moisture (%) in the control group (n=27) and in the β-glucan group (n=30) during the intervention.

10.6 Faecal water geno- and cytotoxicity

The mean percentage of cell survival following FW incubation was 87.8% ±14.6% as assessed by Trypan Blue Dye exclusion assay. Genotoxicity of faecal water samples (TI%>10) at the baseline was assessed in 28 subjects (14 men and 14 women). The selected characteristics of the subjects with genotoxic FW are presented in Table 6.

During the intervention the tail intensity (TI%) in the β-glucan group decreased, reaching statistical significance on the 90th day compared to the control group (p=0.001). The TI% increased 2 weeks after the trial. However, it still remained significantly lower compared to the control group (p=0.048) (Fig. 24). Group specific analysis for the β-glucan group also revealed a significant decrease in the TI% on the 90th day compared to the baseline (p=0.016) (Fig. 24, Table 7).

Table 6. Selected characteristics of volunteers with genotoxic FW (control group, n=14 and β-glucan group, n=14).

	Controls	β-glucan	*p*-value
Age	57.7 ± 5.2	61.4 ± 8.9	0,187
Men/Women *(n)*	7/7	7/7	1,000
BMI	30.18 ± 5.3	27.51 ± 3.4	0.124
Smoking %	64	42	0,256
Pathology (*n*)			
Hyperplastic polyps	1	2	0,541
Serrated adenoma	3	2	0,622
Tubular adenoma	4	5	0,686
Tubulovillus adenoma	4	3	0,663
Tubulovillus adenomas with high-degree dysplasia	2	2	1,000

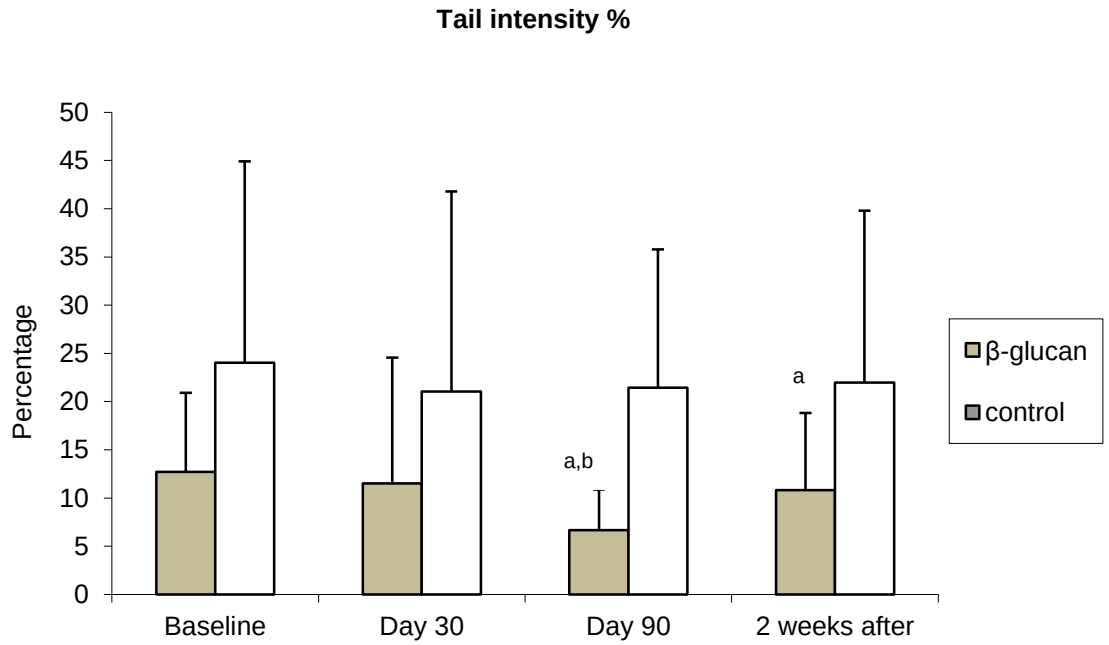


Figure 24. Tail Intensity percent (TI%) of FW in the control group (n=14) and in the β-glucan group (n=14) during the intervention. [a]Significantly different from the control(p<0.05); [b]Significantly different from the baseline(p<0.05).

10.7 Gastrointestinal symptoms

No significant differences were noted in number of evacuations, stool composition, pain, bloating, or in appearance of the blood in the faeces between the β-glucan group and the control group. Flatulence was significantly increased in the β-glucan group on the 90th day of intervention compared to the control (p=0.024). Group specific analysis for the β-glucan group revealed a significantly decreased number of evacuations at the end of the intervention

(Day 90-97) compared to 30th day (p=0.028) (Table 8). One subject failed to return the questionnaire and was therefore excluded from the analysis.

10.8 Nutrition analysis

No significant differences were noted in the energy intake, protein, carbohydrate, saturated, mono-unsaturated or poly-unsaturated fatty acids, fibre, or alcohol intake, or in the BMI between the β-glucan group and the control group. The subjects mean baseline energy intake in this study was 1727 kcal/day (β-glucan group 1646 kcal/day, control group 1807 kcal/day), from which 42.2% was from carbohydrates 16.3% from proteins, 38 % from fat and 3.5% from alcohol intake. Their mean BMI was 28.8kg/m^2 classifying them as overweight (BMI 25-29.9). Group specific analysis for the control group showed a significant decrease in the protein intake at the end of the intervention compared to the baseline (p=0.008) (Table 9). One subject failed to return the questionnaire and was therefore excluded from the analysis.

10.9 Physical activity

The controls were found to be significantly more physically active than subjects in the β-glucan group both before entering (3011.9 vs. 2463.7 kcal/wk, p=0.007) and at the end of the intervention (2978.31 vs. 2413.6 kcal/wk, p=0.004). Both groups scored within moderate exercise level (2000-3500 kcal/wk) (Table 10).

10.10 Physical and mental health

No significant differences were noted in the physical or mental health total scores between the control group and β-glucan group (Table 10).

Table 7. Cytotoxicity (cell survival) and genotoxicity (tail intensity) of FW in 2 feeding groups of polypectomised patients (β-glucan, n=14, control bread, n=14) before, on the 30th day, on the 90th day,and 2 weeks after the dietary intervention.

	Baseline		Day 30		Day 90		2 weeks after		
	Control	β-glucan	Control	β-glucan	Control	β-glucan	Control	β-glucan	*p*-group
Cell survival, %	80.19 ± 24.42	92.22 ± 3.43	89.06 ± 5.29[a]	92.61 ± 2.55	90.58 ± 5.22	93.00 ± 3.60	86.51 ± 23.99	91.79 ± 3.43	0.01
Tail intensity, %	24.05 ± 20.88	12.71 ± 8.19	21.03 ± 20.76	11.52 ± 13.04	21.43 ± 14.34	6.43 ± 3.91[b, c]	21.98 ± 17.82	11.06 ± 8.31[b]	0.01

[a]Significantly different from the β-glucan group (Repeated Measures ANOVA or Friedman test after Bonferroni's adjustment for multiplicity), *p*=0.032.

[b]Significantly different from the control group, p<0.05, [c]Significantly different from the baseline, *p*<0.05.

Table 8. Gastrointestinal symptoms and characteristics of evacuations in the control group (n=27) and in the β-glucan group (n=27) before (Day -7-0), during (Day 30-37), and after the intervention (Day 90-97).

	Day -7-0		Day 30-37		Day 90-97		
7-day symptom score	control	β-glucan	control	β-glucan	control	β-glucan	*p*-group
Abdominal pain	2.04 ± 3.03	2.55 ± 3.93	1.37 ± 2.37	2.69 ± 3.69	2.11 ± 4.69	2.90 ± 4.25	0.256
Bloating	3.44 ± 5.39	5.03 ± 6.90	2.56 ± 4.13	4.62 ± 5.06	3.74 ± 5.56	5.52 ± 6.23	0.158
Flatulence	6.07 ± 5.50	7.55 ± 6.92	6.26 ± 6.16	7.55 ± 6.40	5.74 ± 5.35	9.76 ± 7.32*	0.113
Blood in faeces	0.30 ± 1.03	0.21 ± 1.11	0.37 ± 1.21	0.41 ± 1.57	0.26 ± 1.02	0.66 ± 1.99	0.719
Sum of symptom scores	11.78 ± 11.32	15.21 ± 12.72	10.48 ± 9.71	14.59 ± 10.26	11.74 ± 13.05	18.17 ± 14.9	0.104
No. of evacuations	9.81 ± 4.37	9.30 ± 5.60	9.89 ± 5.39	9.47 ± 4.71	10.07 ± 5.92	8.63 ± 4.16†	0.884
No. of watery evacuations	0.74 ± 1.79	0.21 ± 1.11	0.52 ± 1.74	0.45 ± 1.21	0.56 ± 1.74	0.34 ± 0.99	0.504
Days of diarrhea	0.52 ± 1.45	0.45 ± 1.68	0.30 ± 1.35	0.48 ± 1.21	0.41 ± 1.39	0.24 ± 0.99	0.932

All values are x ± S.D. (Repeated Measures ANOVA or Friedman test after Bonferroni's adjustment for multiplicity).

Symptom intensity was graded as 0=no symptoms, 1-7=mild symptoms, 8-14=moderate symptoms and 15-21=severe symptoms. The possible range for each 7-d symptom score is 0-12 and for total symptom score 0-84. Diarrhea was defined as ≥ 1 watery stool or > 3 stools/day. *Significantly different compared to the control group (*p*<0.05); †Significantly different from the Day 30-37 (*p*<0.05).

Table 9. Nutrition analysis in the β-glucan group (n=29) and in the control group (n=27).

	β-glucan		control		
	baseline	**2 weeks after**	**baseline**	**2 weeks after**	***p*-group**
BMI	27.84 ± 3.68	27.78 ± 3.66	29.81 ± 4.60	29.81 ± 4.52	0.072
Kcal	1645.97 ± 611.53	1667.05 ± 603.61	1807.12 ± 487.94	1704.51 ± 603.61	0.461
Protein %	16.50 ± 3.50	15.81± 2.80	16.01 ± 3.41	14.89 ± 2.91[a]	0.316
Carbohydrate %	41.15 ± 9.47	42.55 ± 9.39	43.25 ± 7.01	45.40 ± 7.66	0.220
Fat, total %	39.41 ± 8.05	39.24 ± 8.41	36.47 ± 5.54	37.09 ± 7.20	0.135
Saturated fat, g	25.13 ± 12.54	25.00 ± 13.78	23.57 ± 11.16	22.67 ± 9.82	0.496
Monounsaturated fat, g	36.25 ± 14.94	35.75 ± 15.80	34.16 ± 10.88	35.18 ± 16.61	0.691
Polyunsaturated fat, g	9.94 ± 4.98	9.28 ± 4.01	10.30 ± 4.44	10.61 ± 5.69	0.426
Alcohol, g	2.90 ± 4.84	2.42 ± 4.91	4.12 ± 4.94	2.79 ± 4.76	0.298
Dietary Fibre total, g	19.32 ± 8.56	18.16 ± 8.71	18.39 ± 10.21	19.19 ± 8.71	0.983

Allvaluesaremean ± S.D.

[a]Significantlydifferentfrom thebaseline (*p*<0.05).

Table 10. Subjects physical activity, and physical and mental health in the β-glucan group (n=30) and in the control group (n=29) at the baseline and after the intervention.

	β-glucan		control		
	baseline	**2 weeks after**	**baseline**	**2 weeks after**	***p*-group**
Physical activity (kcal/d)	2463.70 ± 477.38	2413.57 ± 501.07	3011.93 ± 957.61[a]	2978.26 ± 892.71[a]	0.005[b]
Physical health score	49.05 ± 6.92	48.27 ± 7.56	49.34 ± 8.47	49.07 ± 9.96	0.788
Mental health score	45.73 ± 9.75	45.99± 8.47	45.28 ± 10.43	47.61 ± 11.53	0.815

All values are mean ± S.D.

[a]Significantly different from theβ-glucan group (p<0.05). [b]*p*<0.05.

11. Discussion

Colorectal cancer is the third most common cancer type worldwide and one of the leading causes of death in the USA and Western Europe[Steward and Wild, World Cancer Report 2014]. The intestinal microbiota is considered to play an important role both in colorectal tumour development and in the evolution and modulation of mucosal immunity. The use of functional foods (the oral delivery of probiotics or in combination with prebiotics) that promote the growth of beneficial microbes in the gut, are the basis of dietetic intervention approaches to alter or attenuate intestinal dysbiosis [Tojo *et al.* 2014]. In our placebo-controlled study, an attempt was made to evaluate the effect of barley-derived β-glucan on intestinal microbiota and investigate its involvement in intestinal dysbiosis and in CRC prevention.

We did not record any significant alterations in the bacterial microbiota between the two feeding groups during the three months ingestion of low-dose of (3 g/day) barley-derived β-glucan. Bifidobacteriacounts had a tendency to increase during the intervention. Group specific analysis for bifidobacteria in the β-glucan group showed an increase at the end of the study (90^{th} day) compared to the baseline. However, bifidobacteria were also found increased in the control group at the end of the study compared to the baseline and to the 30^{th} day. Barley β-glucan has been reported to selectively support the growth of bifidobacteria [Shen *et al*. 2012, Mitsou *et al.* 2010, Su *et al.* 2007, Drzikova *et al.* 2005]. Furthermore, the increase of bifidobacteria in the control group could be due to other dietary factors. Bifidobacterial levels have been shown to decrease as total carbohydrate intake decreases [Duncan *et al.* 2007]. The Dietary Guidelines for Americans recommends energy nutrients of total calories to be distributed as follows; 20-35% (average 30%) from fat, 10-35% (average 15%) from protein, and 45-65% (average 55%) from carbohydrates [U.S. Department of Agriculture and U.S. Department of Health and Human Services 2010]. Our subjects' average carbohydrate intake was relatively low, only 41% of total energy.

In addition, in the β-glucan group we noticed a decrease in enterococci 2 weeks after the intervention compared to the 90th day.Also, in the control group enterococci decreased on the 90^{th} day, and 2 weeks after the intervention compared to the baseline. Enterococcal strains have a poor ability to utilize β-glucooligomers [Crittenden *et al.* 2002]. However, this may not explain the result since the decrease occurred 2 weeks after the subjects finished consuming β-glucan enriched bread. Enterococci counts have shown to decrease as fibre intake increases. In mice, skipping from plan oligosaccharides rich diets into a high-fat and high-sugar dietsignificantly increased the enterococcal levels [Turnbaugh *et al.* 2009]. Enterococci, such as *Enterococcus faecalis,* are pro-inflammatory opportunistic pathogens in

human intestinal microbiota. They are considered to be driver bacteria for CRC by producing extracellular superoxide, which can lead to epithelial cell damage and increased turnover [Balamurugan *et al.* 2008, Huycke *et al.* 2002] and also promote chromosome instability via macrophage-induced bystander effects [Wang *et al.* 2012a, Wang *et al.* 2007]. CRC patients, with respect to healthy controls, are significantly enriched in *Enterococcaceae*, especially in *E. faecalis*, which is also a known 'driver bacteria' in CRC development [Balamurugan *et al.* 2008, Wang *et al.* 2007, Tjasma*et al.* 2012]. By increasing our subjects' fibre intake we may have also decreased the count of these opportunistic pathogenic bacteria.

Barley β-glucan has been reported to selectively support the growth of lactobacilli [Shen *et al.* 2012, Su *et al.* 2007, Snart *et al.* 2006]. In our study the barley β-glucan feeding regime did not significantly favour the growth of lactobacilli. Only a small non significant increase was noted in the β-glucan group on the 90th day of the study compared to the 30th day. Data from several studies demonstrate that results depend on the source, dosage, viscosity, molecular weight, and food processing of β-glucan. The higher the percentage of in-soluble fibre vs. soluble fibre, the higher the viscosity, the lower the molecular weight and the higher the dosage, the better the effects [Arena *et al.* 2014, Shen *et al.* 2012, Lazaridou and Biliaderis 2007, Snart *et al.* 2006]. During our intervention, the subjects were given 3g/ barley β-glucan/day. Perhaps this was not enough to induce a more significant increase in lactobacilli levels.

In our study, β-glucan administration in polypectomised patients induced no changes in both SCFAs acetate or propionate concentration during the intervention. An increase was recorded on butyrate concentration in the control group at the end of the intervention compared to the β-glucan group. We also noted an increase in the valerate concentration and in the sum of other SCFAs (valerate + caproate + heptanoate) concentrations on the 90th day compared to the control group.One important factor that maybe affecting our results is the subjects' age. Microbiota of subjects over 60 years old has often an altered bacterial composition towards a more proteolytic one. This change consists of an increase in proteolytic bacteria and a decrease in saccharolytic bacteria [Bischoff 2016, Mäkivuokko *et al.* 2010, Woodmansey 2007]. Interestingly, CRC incidence is strongly related to age, with the highest incidence rates being in older men and women [Cancer research UK 2015].Valerate like other minor SCFAs are metabolites from the bacterial degradation of proteins. Known butyrate producers, such as *Clostridium* cluster XIVa and *Faecalibacterium prausnitzii*, have also been observed at lower levels in older volunteers [Mueller *et al.* 2006, Rajilic-Stojanovic *et al.* 2009].

Other possible reasons for the observed decrease in the butyrate concentration could be impaired metabolisms of SCFA due to physical condition, such as dysbiosisin microbiota and the amount of available substrate (insufficient amount of β-glucan). Increased intake of butyrate (extra requirement) or poor uptake of butyrate by colonic cells, or even high entry baseline levels can affect the end result [Vernia *et al.* 1988a+b, Hallert *et al.* 2003, Gonçalves and Martel 2013, McOrist *et al*. 2011, Nilsson *et al.* 2008].

Studies have shown that SCFA excretion in human faeces is influenced by the presence of fibre sources in the diet. In another β-glucan study, 8 weeks administration of β-glucan-enriched oat bran in healthy subjects increased the concentration of acetic, propionic, butyric, iso-butyric, and iso-valeric acid. The concentration of these carboxylic acids did not increase until after 8 weeks indicating that a long time was required in order to change the balanced composition of colonic microbiota and increase the number of bacteria that can ferment the increased amount of available substrate [Nilsson *et al*. 2008]. The three-month intervention time should have been long enough to observe the changes. One likely reason could be that 3g/day of barley-derived β-glucan was not sufficient to affect the concentration of these SCFAs. In the study by Nilsson *et al.* subjects were given 40 g of oat bran enriched with 10g of β-glucan daily.

Interestingly, our study is not the first human prebiotic study reporting a decrease in butyrate concentration. In a short interventional study, 12 healthy adults were fed fructo-oligosaccharides and maltodextrin for two weeks. After the ingestion, an alteration in bacterial fermentation was discovered, such as increased percentage of acetate, and decreased percentage of butyrate concentration [Scholtens *et al.* 2006].

Physical condition can influence the SCFA metabolism. Impaired metabolisms of SCFA have been reported in inflammatory bowel disease [Vernia *et al.* 1988a+b]. The SCFA concentration from FW of 62 ulcerative colitis patients showed that SCFA levels were high in the inactive and mild phase, but significantly decreased in the severe phase of the disease [Vernia *et al.* 1988a]. In another study, in order to measure and compare SCFA concentrations, a 24-h faecal sample collection was conducted in patients with ulcerative colitis, or Crohn's disease, and in healthy controls. SCFA concentrations, especially butyrate, were found in significantly lower levels in ulcerative colitis patients compared to the controls or to the Crohn's disease patients [Vernia *et al.* 1988b]. Lower baseline concentration of butyric acid in UC patients' compared to healthy controls has also been reported [Hallert *et al.* 2003]. Butyrate is known to be transported into colonic epithelial cells by two specific carrier-mediated transport systems, the monocarboxylate transporter 1 (MCT1) and the sodium-coupled monocarboxylate transporter 1 (SMCT1). *In vitro* studies with colonic epithelial

cancer cells have shown a reduction in butyrate uptake through a reduction in MCT1 and SMCT1 protein expression [Gonçalves and Martel 2013]. Perhaps high-risk CRC patients also have some level of impairment in their SCFA metabolism.

A considerable high baseline butyrate level could also affect the outcome. In another study, when resistant starch was administrated to healthy males and females, butyrate levels were shown to increase in response to administration in most individuals but often decreased when entry butyrate levels were high. The baseline butyrate concentrations ranged from 3.5 to 32.6 mmol/kg (mean 13.6 mmol/kg) [McOrist *et al*. 2011]. Similar concentrations for n-butyric acid were measured in healthy subjects; 4.0-53.0 mmol/kg (mean 12.4 mmol/kg) [Høverstad *et al.* 1984]. In our study, the recorded baseline butyrate concentration varied from 4.2 to 115.9 mmol/kg and the average butyrate concentration was much higher than the one in the studies mentioned above (mean 35.5 mmol/kg). In addition, our results showed large inter-individual variation.Most of the SCFA formed in the human colon are absorbed and it may be argued that faecal concentrations are a complex function of rate of formation, absorption and utilization [Nilsson *et al.* 2008]. One could claim that extra butyrate was utilized before reaching the faeces or that the butyrate concentration was already at its peak level.

Although butyrate is widely studied and established for its anticarcinogenic properties, a variety of other potential SCFAs or medium chain fatty acids also exist in the colon. A three-month β-glucan intervention resulted in an increase in the valerate concentration, and in the sum of other SCFAs (valerate + caproate + heptanoate) concentrations on the 90^{th} day compared to the control group. Valerate is a straight-chain fatty acid and a metabolite from bacterial degradation of protein. In a few *in vitro* studies valerate demonstrated a potential anticarcinogenic ability. Valerate together with propionate caused growth arrest and differentiation in human colon carcinoma cells, due to their antimicrotubule agent activity, blocking cell growth by stopping mitosis in cancer cells [Hinnebusch *et al.* 2002, Kilner *et al.* 2012 and 2016].

In our study, the increase observed regarding the concentration of other SCFA (valerate + caproate + heptanoate) seen on the 90^{th} day, probably resulted also from an increase in caproate concentration. The origin of this fatty acid is less known, but there is evidence suggesting it may also belong to the protein-derived group together with the other minor acids [Siigur *et al.* 1994]. The possible role of this fatty acid in CRC prevention is unknown. While, as mentioned above, valerate together with propionate caused growth arrest and differentiation in human colon carcinoma cells, caproate failed to have any appreciable effects on cell growth or differentiation [Hinnebusch *et al.* 2002]. When the anticancer properties of

capric, caprylic, and caproic acids on human colorectal, skin, and mammary gland cancer cells were investigated, caproate reduced cancer cell viability by 70% to 90% compared to controls, showing a prospective anticancer effect [Narayanan *et al.* 2015].

As mentioned before, the Dietary Guidelines for Americans recommend energy nutrients of total calories to be 10-35% (average 15%) from protein [U.S. Department of Agriculture and U.S. Department of Health and Human Services 2010]. Our subjects' average protein intake was 16% suggesting that the increase in valerate, iso-valerate and sum of other SCFA did not result from a protein rich diet. On the other hand, our subjects' average carbohydrate intake was relatively low, only 41%, indicating that their diets were high in fat. Reduced-carbohydrate, high-protein weight-loss diets were investigated in obese subjects for their effect on butyrate-producing bacteria. Despite the desired weight loss, subjects had reductions in SCFAs, especially butyrate, which corresponded to a reduction in the *Roseburia*/*E. rectal* group [Duncan *et al.* 2007, Russell *et al.* 2011]. The higher protein intake increased the proportions of branched-chain fatty acids [Russell *et al.* 2011] and the beneficial Bifidobacteria was shown to decrease as carbohydrate intake decreased [Duncan *et al.* 2007].

B-glucan administration did not have any impact on faecal enzyme activity during the trial. Little is known to date on whether the activity of these enzymes varies in response to changes in dietary intakes or to the exposure to dietary glycosides reaching the colon [McIntosh *et al.* 2012]. B-glucuronidase is generated by a wide range of gut bacteria including *Escherichia coli*, and some species of *Bacteroides* and *Clostridium* [Gloux *et al.* 2011]. Lactobacilli and bifidobacteria are known for their high β-glucosidase activity [Saito *et al.* 1992, Rowland and Tanaka 1993, Otieno *et al.* 2005]. Both oat β-glucan and barley β-glucan administration significantly decreased β-glucuronidase activity in rat faeces compared to the control group and this was attributed to cereal β-glucans acting as a prebiotic selectively promoting the growth of *Bifidobacterium* spp. [Shen *et al.* 2012]. Oligosaccharides have the ability to increase the activity of β-glucosidase, which isusually attributed to the ability of oligosaccharides to stimulate the growth of lactic acid bacteria [Saito *et al.* 1992, Rowland and Tanaka 1993]. Digestion of different probiotic bacteria are also known to enhance the activity of this enzyme [Verma and Shukla 2013, de Preter *et al.* 2008].B-glucosidase produced by probiotics (mainly by lactobacilli) has been found to prevent colon cancer by releasing flavonoids [Wollowski *et al.* 2001].In our study we did not record any changes in β-glucuronidase or in β-glucosidase activity. Interestingly, no significant changes in bifidobacteria and/or lactobacilli counts or in the *Escherichia coli*, *Bacteroides*, and *Clostridium* countswere detected between the feeding groups.

Faecal water contains bioactive compounds originated from dietary intake, such as bile acids, fatty acids, N-nitroso compounds, and HCAs, which are potentially related to CRC

initiation and development. It could be thus proposed as an additional biomarker, given that it can be collected easily in a non-invasive manner. Genotoxicity of FW is one of the biological activities which have been widely studied in colonic cell lines using the single cell gel electrophoresis (COMET) assay [Pearson *et al.* 2009].

The impact of synbiotics among various cancer risk parameters, such as the genotoxicity of FW, was investigated on polypectomised and cancer patients. However, no difference in the incidence of FW genotoxicity between the two groups was reported at the intervention baseline [Rafter *et al.* 2007]. In our study, almost half (49%) of the baseline FW samples from polyectomised patients were found to be genotoxic. It seems that the incidence of FW genotoxicity is not differentiated from the genotoxicity detected in healthy Swedish, English, or Irish volunteers [Venturi *et al.* 1997, Woods *et al.* 2002]. In another COMET assay study, while measuring the FW genotoxicity of adenoma patients and controls on human colon carcinoma cells, no differences were found between the two groups [Nordling *et al.* 2003].

Unexpectedly, there was a noticeable difference in FW genotoxicity between the control group and the β-glucan group throughout the study, albeit this was not significant ($p=0.070$) (Fig. 12). There were differences (non-significant) in the smoking habits, age, and BMI between the two groups in our study. This might have been the cause of the observed discrepancy. For example, 64% of the controls were smokers compared to 42% in β-glucan group. Smoking is known to increase the exposure to DNA damage and to deteriorate the benefit from a dietary intervention on cancer prevention [Glei *et al.* 2005]. Three months ingestion of low-dose (3g/day) barley-derived β-glucan led to a significant decrease in FW genotoxicity between the two feeding groups of polypectomised patients. In the β-glucan group the FW genotoxicity decreased (gradually during the intervention and partially recovered during the wash-out period) compared to the baseline. In contrast no alterations were observed in the control group throughout the intervention study.

To our knowledge this is the first study reporting an antigenotoxic action of cereal β-glucan in a high CRC risk group. In some of our samples we noticed a high inter-individual variation. Perhaps a more controlled diet, longer intervention duration, or a 24h total faecal sample collection could have reduced this variation. The variability of the faecal samples' genotoxicity is one of the issues frequently discussed, as it is often appears among faecal samples and often prevents reaching statistical significance [Gratz *et al.* 2011, Windey *et al.* 2015].

In vitro chemoprotective activity of barley β-glucan has been previously reported against chemical mutagens, although in different cell lines [Oliveira *et al.* 2006, Angeli *et al.* 2009]. Furthermore, barley-derived β-glucan was recently found to be dose-dependently both geno-

and cytoprotective in broiler chicken lymphocytes exposed to increasing concentrations of aflatoxin B1 [Zimmermann *et al.* 2015].

Barley β-glucan has been previously proposed to exhibit potential prebiotic action due to changes in the gut microbiota of healthy subjects [Mitsou *et al.* 2010]. Various trials with prebiotics in healthy volunteers have examined their role on the genotoxicity of FW with differentiated conclusions. In a study with healthy volunteers over 50 years old, the intervention with galacto-oligosaccharides (GOS) had a bifidogenic effect on the gut microbiota, but did not change the FW genotoxicity [Walton *et al.* 2012]. Similar results were reported in a randomized controlled trial in healthy subjects where the administration of wheat bran extracts altered the microbial composition of the gut but did not affect faecal genotoxicity [Windey *et al.* 2015]. In contrast, an intervention with polydextrose in healthy subjects significantly reduced FW genotoxicity and provoked changes in the gut microbiota. In the same trial changes in the subject's bowel habits were also reported (subjects with genotoxic FW). Polydextrose consumption resulted in less abdominal discomfort and there was a trend for less hard and more formed stools [Costabile *et al.* 2012].

The mechanisms involved in the reduction of FW genotoxicity by prebiotics are not well known. Prebiotics are known to selectively stimulate the growth and activity of a limited number of colonic bacteria (especially lactic acid-producing bacteria) [Gibson and Roberfroid 1995]. These bacterial strains could inactivate genotoxins in FW [Burns and Rowland 2004]. It has been suggested that the inactivation could be due to the fermentation products of probiotics and prebiotics' improving barrier function in Caco-2 monolayers [Commane *et al.* 2005, Rafter *et al.* 2007, Stewart and Wild 2014].

In our study the subjects' perspective view of the quality of life was measured by the use of SF-36 and gastrointestinal symptom questionnaires. Except for the increase in flatulence score at the end of the intervention (Day 90-97), we did not notice any other significant differences in the gastrointestinal symptoms scores between the β-glucan group and control group. It is common in studies administering prebiotics to have an increase in bloating and/or flatulence [Bouhnik *et al.* 2007, Mitsou *et al.* 2009, François *et al.* 2014], possibly due to increased fibre intake. In our earlier interventional studies on polypectomised patients and on healthy volunteers, β-glucan administration resulted in decreased bloating [Turunen *et al.*2011] and decreased abdominal pain score [Mitsou *et al.* 2010, Turunen *et al.* 2011]. Fairtolerance could be also due to the low dose of β-glucan administrated. Group specific analysis for β-glucan revealed a significantly decreased number of evacuations at the end of the intervention (Day 90-97) compared to the baseline. Cereal β-glucans are soluble fibres and their consumption does not usually enhance the number of faecal evacuations [Ibrügger *et*

*al.*2013].Normal stool frequency is considered to be between 1 to 2 bowel movements per day [Rao *et al.* 2011]. Subjects in the β-glucan group had an average number of evacuations 1.6 ± 0.8 times per day at the beginning of the study (control: 1.5 ± 0.8 times/day). This decreased into 1.3 ± 0.7 times per day at the end of the study (control: 1.7 ± 1.0 times/day). Even though the change was significant, it is still a pretty normal evacuation number. During the trial we did not record any significant change in the consistency of evacuations of the faeces, indicating that this effect was not due to constipation. Furthermore, when transit time is already optimal, i.e., between 24 and 48 h, additional dietary fibre would not be expected to increase it [Rao *et al.* 2011]. Intestinal polyps, unlike other GI disorders such as inflammatory bowel disease, are usually asymptomatic in the patient's colon. Yet, quality of life plays a significant role for patients with GI disorder. However, during our intervention we did not record any changes in the quality of life.

There is strong evidence suggesting that physical inactivity and obesity increases the risk of many adverse health conditions, such as colorectal cancer [Lee *et al.* 2012, Zackular *et al.* 2014]. According to the data from our physical activity questionnaires, even though both groups were within the moderate exercise level score, subjects in the control group were physically more active than the subjects in the β-glucan group. No significant differences were detected in the living conditions of our subjects (city vs. country, working conditions, exposure to exercise programs) between the two groups. We suppose that this difference in activity level was due to a small difference in age between the study groups. Subjects in the β-glucan had an average age over 62 years-old (β-glucan group 62.4 ± 8.7 vs. control group 59.8 ± 7.3). This means that the percentage of pensioners was higher in the β-glucan group. In our study, according to the baseline BMI, our subjects were overweight in both feeding groups (β-glucan group 27.8, control group 29.8). Men, Caucasians, and those with a higher BMI are more likely to have colorectal cancer [Zackular *et al.* 2014].

It is generally known that a high intake of dietary fibre can protect against the development of colorectal cancer [Anderson *et al.* 1995]. Inadequate dietary fibre intake is a global concern, as average intakes are well below recommendations across many countries [World Health Organization and Food and Agricultural Organization of the United Nations 2003, de Vries *et al.* 2015]. Barley and other cereals are a rich source of fibre, inexpensive, easy to prepare, highly nutritious, and easily included in our everyday diet [Bansal *et al.* 1977].

12. Conclusions

(1) B-glucan ingestion in polypectomised patients was practically well tolerated.
(2) B-glucan ingestion resulted in an increase in bifidobacteriacountsand an increase, although non-significant in lactobacilli levels. However, bifidobacteria were also found increased in the control group. A higher daily dose of β-glucan and/or a higher number of participants in the trial could probably have resulted in a more profound prebiotic effect.
(3) Regarding the SCFAs measured, valerate and the sum of other SCFAs concentrations were increased during the intervention in polypectomised patients. It is unknown whether this was due to subjects more proteolytic microbiota or barley β-glucans ability to increase their production.
(4) B-glucan ingestion in polypectomised patients significantly decreased the FW genotoxicity. This effect could be due to barley β-glucans' ability to stimulate the growth and activity of beneficial intestinal bacteria, to act as an antioxidant trapping free-radicals,to act as immunomodulators interacting with immune cells, or barley β-glucanscould interfere with the expression of genes that code for enzymes responsible for xenobiotic-metabolizing.

Our findings overall suggest that β-glucan consumption could possibly provide protection against colon cancer initiation.

13. Future studies

CRC is associated with oxidative stress, and assessment of oxidative stress along with the possible administration of antioxidants is important for the treatment and prevention of colorectal cancer**[**Chang *et al.* 2008]. The data from COMET-assay showed a decrease in the FW genotoxicity. Based on these results barley β-glucan could have a possible role as an antioxidant. COMET-assay measures various types of DNA damage, including oxidative stress. In addition, it is documented that CRC risk is influenced by microbial composition, showing differences according to disease progression step and tumour severity [Mira-Pasqual *et al.* 2015]. For an enhanced understanding of the underlying dysbiosis of microbiota and shifts in microbial composition after the intervention there is a need to further analyse our samples by using the latest sequencing techniques. In order to validate these encouraging results and further elucidate the role of β -glucan ingestion in the multistage development of this malignant disease, mechanistic as well as larger epidemiological studies are needed.

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Attachment 1. Gastrointestinal symptoms questionnaire

ΤΟ ΠΑΡΟΝ ΦΥΛΛΑΔΙΟ ΠΕΡΙΕΧΕΙ ΕΡΩΤΗΣΕΙΣ, ΟΙ ΑΠΑΝΤΗΣΕΙΣ ΤΩΝ ΟΠΟΙΩΝ ΕΙΝΑΙ ΣΗΜΑΝΤΙΚΕΣ ΓΙΑ ΤΗΝ ΟΛΟΚΛΗΡΩΜΕΝΗ ΕΙΚΟΝΑ ΤΗΣ ΚΛΙΝΙΚΗΣ ΣΑΣ ΚΑΤΑΣΤΑΣΗΣ. ΣΑΣ ΠΑΡΑΚΑΛΟΥΜΕ ΝΑ ΑΠΑΝΤΗΣΕΤΕ ΣΕ ΟΛΕΣ ΤΙΣ ΕΡΩΤΗΣΕΙΣ. ΟΛΕΣ ΟΙ ΑΠΑΝΤΗΣΕΙΣ ΘΑ ΠΑΡΑΜΕΙΝΟΥΝ ΑΥΣΤΗΡΩΣ ΕΜΠΙΣΤΕΥΤΙΚΕΣ. ΕΥΧΑΡΙΣΤΟΥΜΕ ΓΙΑ ΤΗ ΣΥΜΜΕΤΟΧΗ ΣΑΣ.

ΟΔΗΓΙΕΣ

ΤΟ ΦΥΛΛΑΔΙΟ ΑΥΤΟ ΘΑ ΧΡΕΙΑΣΤΕΙ ΝΑ ΣΥΜΠΛΗΡΩΘΕΙ ΣΥΝΟΛΙΚΑ ΤΡΕΙΣ ΦΟΡΕΣ ΚΑΤΑ ΤΗ ΔΙΑΡΚΕΙΑ ΤΗΣ ΜΕΛΕΤΗΣ (ΠΡΙΝ ΤΗΝ ΠΑΡΕΜΒΑΣΗ, ΤΟΝ ΤΡΙΤΟ ΜΗΝΑ, ΚΑΙ ΜΕΤΑ ΑΠΟ ΤΗΝ ΠΑΡΕΜΒΑΣΗ). ΣΥΓΚΕΚΡΙΜΕΝΑ, ΤΙΣ ΔΕΔΟΜΕΝΕΣ ΧΡΟΝΙΚΕΣ ΣΤΙΓΜΕΣ ΘΑ ΣΥΜΠΛΗΡΩΝΕΤΑΙ ΚΑΘΗΜΕΡΙΝΑ ΓΙΑ ΜΙΑ ΕΒΔΟΜΑΔΑ (ΗΜΕΡΑ 1, ΗΜΕΡΑ 2 …).

ΕΡΩΤΗΣΗ 1

ΚΑΤΑ ΤΗ ΔΙΑΡΚΕΙΑ ΤΗΣ ΗΜΕΡΑΣ ΠΟΣΕΣ ΚΕΝΩΣΕΙΣ ΕΧΕΤΕ; ΑΝ ΔΕΝ ΥΠΑΡΧΕΙ ΚΕΝΩΣΗ ΚΥΚΛΩΝΕΤΕ ΤΟ 0 ΚΑΙ ΣΥΝΕΧΙΖΕΤΕ ΜΕ ΤΗ ΣΕΛΙΔΑ ΠΟΥ ΑΦΟΡΑ ΤΗΝ ΕΠΟΜΕΝΗ ΗΜΕΡΑ.

ΕΡΩΤΗΣΗ 2

ΟΙ ΕΡΩΤΗΣΕΙΣ ΑΥΤΕΣ ΑΦΟΡΟΥΝ ΤΗΝ ΟΨΗ ΤΩΝ ΚΟΠΡΑΝΩΝ. ΤΟ 0 ΑΝΑΦΕΡΕΤΑΙ ΣΤΗΝ ΑΡΙΣΤΕΡΗ ΠΕΡΙΓΡΑΦΗ (ΥΔΑΡΑ) ΚΑΙ ΤΟ 4 ΣΤΗΝ ΔΕΞΙΑ (ΣΚΛΗΡΑ).

ΕΡΩΤΗΣΕΙΣ 3 ΕΩΣ 6

ΠΕΡΙΓΡΑΦΟΥΝ ΤΑ ΣΥΜΠΤΩΜΑΤΑ ΜΕ ΜΙΑ ΚΛΙΜΑΚΑ ΣΟΒΑΡΟΤΗΤΑΣ ΑΠΟ ΤΟ 0 (ΑΣΥΜΠΤΩΜΑΤΙΚΑ) ΜΕΧΡΙ ΤΟ 4 (ΣΟΒΑΡΑ).

ΓΙΑ ΤΥΧΟΝ ΣΥΜΠΛΗΡΩΜΑΤΙΚΕΣ ΠΛΗΡΟΦΟΡΙΕΣ ‘Η ΔΙΕΥΚΡΙΝΗΣΕΙΣ ΜΠΟΡΕΙΤΕ ΝΑ ΕΠΙΚΟΙΝΩΝΗΣΕΤΕ ΜΕ ΤΟ ΧΑΡΟΚΟΠΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ, ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΔΙΑΙΤΟΛΟΓΙΑΣ ΚΑΙ ΔΙΑΤΡΟΦΗΣ. ΤΗΛ. 210-9549135 ‘Η 69-37160509.

ΗΜΕΡΑ 1

1. ΠΟΣΟ ΣΥΧΝΑ ΕΧΕΤΕ ΚΕΝΩΣΕΙΣ ΤΗΝ ΗΜΕΡΑ;

0 ΚΑΜΙΑ ΚΕΝΩΣΗ
1 ΜΙΑ ΦΟΡΑ ΤΗΝ ΗΜΕΡΑ
2 ΔΥΟ ΦΟΡΕΣ ΤΗΝ ΗΜΕΡΑ
3 ΤΡΕΙΣ ΦΟΡΕΣ ΗΜΕΡΗΣΙΩΣ
4 ΤΕΣΣΕΡΙΣ Η ΠΑΡΑΠΑΝΩ ΦΟΡΕΣ ΤΗΝ ΗΜΕΡΑ

2. ΤΙ ΣΥΣΤΑΣΗ ΕΧΟΥΝ ΤΑ ΚΟΠΡΑΝΑ ΣΑΣ;

ΥΔΑΡΑ 0 1 2 3 4 ΣΚΛΗΡΑ

3. ΥΠΑΡΧΕΙ ΑΙΜΑ ΣΤΑ ΚΟΠΡΑΝΑ ΣΑΣ;

ΧΩΡΙΣ ΑΙΜΑ 0 1 2 3 4 ΠΟΛΥ ΑΙΜΑ

4. ΕΧΕΤΕ ΚΟΙΛΙΑΚΟ ΑΛΓΟΣ ΚΑΤΑ ΤΗ ΔΙΑΡΚΕΙΑ ΤΗΣ ΗΜΕΡΑΣ;

ΧΩΡΙΣ ΑΛΓΟΣ 0 1 2 3 4 ΈΝΤΟΝΟ ΑΛΓΟΣ

5. ΕΧΕΤΕ ΚΟΙΛΙΑΚΗ ΔΙΟΓΚΩΣΗ ΚΑΤΑ ΤΗ ΔΙΑΡΚΕΙΑ ΤΗΣ ΗΜΕΡΑΣ;

ΧΩΡΙΣ ΔΙΟΓΚΩΣΗ 0 1 2 3 4 ΈΝΤΟΝΗ ΔΙΟΓΚΩΣΗ

6. ΕΧΕΤΕ ΜΕΤΕΩΡΙΣΜΟ ΚΑΤΑ ΤΗ ΔΙΑΡΚΕΙΑ ΤΗΣ ΗΜΕΡΑΣ;

ΧΩΡΊΣ ΜΕΤΕΩΡΙΣΜΌ 0 1 2 3 4 ΈΝΤΟΝΟΣ ΜΕΤΕΩΡΙΣΜΌΣ

Attachment 2.Nutrition analysis questionnaire

ΗΜΕΡΟΛΟΓΙΟ
ΚΑΤΑΓΡΑΦΗΣ ΤΡΟΦΙΜΩΝ

Όνομα .....

Κωδικός .....

Εργαστήριο Διατροφής και Κλινικής Διαιτολογίας
Τμήμα Επιστήμης Διαιτολογίας - Διατροφής
Χαροκόπειο Πανεπιστήμιο

Ευχαριστούμε για τη βοήθειά σου στην έρευνα αυτή.

Οδηγίες για να συμπληρώσεις αυτό το ημερολόγιο

- Γράψε **ΟΛΑ** τα τρόφιμα (φαγητά και ποτά) που θα φας τις επόμενες ____ ημέρες.
  Για κάθε ημέρα ξεκίνα από μια καινούργια σελίδα.
  Χρησιμοποίησε όσες σελίδες χρειάζεσαι για κάθε ημέρα.
- Σημείωσε την ώρα που άρχισες να τρως το φαγητό ή το κολατσιό σου.
- Σημείωσε την εμπορική επωνυμία (μάρκα) του τροφίμου αν τη θυμάσαι.
- Μην αλλάξεις τις συνήθειες του φαγητού σου και τη δίαιτά σου επειδή συμπληρώνεις αυτό το ημερολόγιο.
- Προσπάθησε να είσαι όσο το δυνατόν πιο σαφής στις περιγραφές των τροφίμων.
  Για παράδειγμα ***Αντί για*** σαλάτα ***καλύτερα να γράψεις*** σαλάτα μαρούλι

κρέας	χοιρινή μπριζόλα ψητή
τοστ	τοστ με ζαμπόν, τυρί, τομάτα

  Μην ξεχάσεις να γράψεις: τα διάφορα *"σνακ"*, τα τσιμπολογήματα μεταξύ των γευμάτων, τα ροφήματα *(καφέδες κ.λπ.)*, τα αναψυκτικά, τις τσίχλες και τις καραμέλες, τους ξηρούς καρπούς.
  Επίσης, μην ξεχάσεις τα συμπληρώματα διατροφής, όπως οι βιταμίνες.
- Είναι πολύ σημαντικό να γράψεις σωστά τις ποσότητες αυτών που έφαγες.
  Για το λόγο αυτό διάβασε προσεκτικά τις παρακάτω οδηγίες.
  Πρόσεξε: Σημείωσε μόνο την ποσότητα του φαγητού που πραγματικά έφαγες, και όχι ό,τι περίσσεψε στο πιάτο.

Γάλα - γιαούρτι: Υπολόγισε την ποσότητα, χρησιμοποιώντας ως "μεζούρα" το ποτήρι, το φλιτζάνι και το κεσεδάκι ή γράψε άλλη τυποποιημένη συσκευασία. Μην ξεχάσεις να σημειώσεις την περιεκτικότητα σε λιπαρά *(πλήρες, 1,5% λιπαρά, άπαχο, κλπ.)*, αν είναι σοκολατούχο ή αν περιέχει φρούτα (π.χ. *γιαούρτι με κομμάτια ροδάκινο*).

Δημητριακά πρωινού: Υπολόγισε την ποσότητα σε κουταλιές της σούπας ή φλιτζάνια του τσαγιού. Μην ξεχάσεις να σημειώσεις το είδος (π.χ. *κορν φλέικς, κουάκερ, κλπ.*), τη ζάχαρη που έβαλες και βέβαια το γάλα (*βλ.* παραπάνω).

Ψωμί - φρυγανιές - αρτοσκευάσματα: Σημείωσε το είδος: *ψωμί άσπρο, μαύρο, ολικής αλέσεως, στρογγυλό ψωμάκι, κουλουράκι με σουσάμι,* κλπ. Γράψε την ποσότητα που έφαγες σε φέτες (μια φέτα σαν αυτή του ψωμιού για τοστ) ή κομμάτια, π.χ. *1 φέτα ψωμί άσπρο, 3 φρυγανιές σικάλεως, 2 κράκερς, 1 κουλουράκι με σταφίδες,* κλπ.

Ζυμαρικά - ρύζι *(μαγειρεμένα)*: Υπολόγισε την ποσότητα χρησιμοποιώντας ως "μεζούρα" το φλιτζάνι του τσαγιού.

Τυρί: Γράψε το είδος (π.χ. *κασέρι, γραβιέρα, φέτα, κλπ.*) και την ποσότητα χρησιμοποιώντας ως "μεζούρα" τη φέτα τυριού για τοστ.

Αυγά: Γράψε τον αριθμό και τον τρόπο μαγειρέματος (π.χ. *2 αυγά τηγανιτά, ομελέτα ή βραστά*).

Κρέας - κοτόπουλο - ψάρι: Σημείωσε το είδος γράφοντας όσο μπορείς πιο αναλυτικά την ποσότητα, το μέγεθος και τον τρόπο μαγειρέματος:

π.χ. 1 *μεγάλη* μπριζόλα χοιρινή *ψητή στα κάρβουνα*
ή 2 *μικρά* μπαρμπούνια *τηγανιτά*
ή 1 *μεσαίο* μπούτι κοτόπουλου *ψητό στο φούρνο*

Προσοχή: Αν είναι μαγειρεμένο μαζί με κάτι άλλο, π.χ. κοτόπουλο με πατάτες στο φούρνο, γράψε ξεχωριστά για τις πατάτες (βλ. ***Σύνθετα φαγητά***).

Όσπρια - σούπες: Γράψε πόσα βαθιά πιάτα, ή πόσα φλιτζάνια του τσαγιού ή κουταλιές της σούπας έφαγες (π.χ. *1 πιάτο φακές, 1 βαθύ πιάτο ψαρόσουπα, 3 κουταλιές της σούπες ρεβύθια*).

Λαχανικά - σαλάτες: Υπολόγισε την ποσότητα χρησιμοποιώντας ως "μεζούρα" το φλιτζάνι του τσαγιού, την κουταλιά της σούπας ή απλά γράψε τον αριθμό. Μην ξεχάσεις να σημειώσεις το μέγεθος καθώς και το αν τα λαχανικά είναι φρέσκα ή έχουν μαγειρευθεί και πώς (π.χ. *1 φλιτζάνι λάχανο σαλάτα, 2 μεγάλα καρότα ωμά, 5 κομμάτια μεσαίου μεγέθους πατάτες φούρνου κλπ.*).

Για τα τηγανιτά λαχανικά (π.χ. *τηγανιτές πατάτες, κολοκυθάκια κλπ.*) γράψε αν έφαγες πέντε, δέκα, είκοσι ..... ή γράψε την ποσότητα σε μερίδες (*1 μερίδα fast food*).

Φρούτα: Σημείωσε το είδος, τον αριθμό και το μέγεθος (π.χ. *2 μεγάλα μήλα, ½ φέτα πεπόνι, 12 ρώγες σταφύλι*). Μην ξεχάσεις να διευκρινίσεις αν το φρούτο είναι φρέσκο ή κονσέρβα.

Χυμοί φρούτων - Αναψυκτικά: Υπολόγισε την ποσότητα χρησιμοποιώντας ως "μεζούρα" το ποτήρι ή το κουτάκι της συσκευασίας *(330 ml)*. Διευκρίνισε αν ο χυμός είναι φρέσκος ή τυποποιημένος καθώς και το είδος του αναψυκτικού (με ή χωρίς ανθρακικό, light).

Άλλα ποτά: Γράψε πόσα ποτά ήπιες (π.χ. *1 βότκα πορτοκάλι*) ή υπολόγισε την ποσότητα σε ποτήρια (μικρά - μεγάλα), μπουκάλια ή κουτάκια (π.χ. *1 ποτηράκι κρασί κόκκινο, 1 μπουκάλι μπύρα κλπ.*).

Γλυκά - σνακ: Για τα γλυκά *(σοκολάτες, μπισκότα, παγωτά, κλπ.)* και τα σνακ *(τυρόπιτες, μπουγάτσες, κλπ.)* χρησιμοποίησε ως "μεζούρα" το φλιτζάνι του τσαγιού, τον αριθμό των κομματιών ή γράψε την τυποποιημένη ποσότητα (π.χ. *1 ξυλάκι παγωτό κρέμα, 1 φλιτζάνι παγωτό παρφέ, ½ πάστα σοκολατίνα, 1 μεγάλη τυρόπιτα, 1 μικρό σακκουλάκι πατατάκια κλπ.*).

Ζάχαρη - μέλι - μαρμελάδα: Υπολόγισε την ποσότητα σε κουταλάκια του γλυκού ή της σούπας.

Λάδι - βούτυρο: Υπολόγισε την ποσότητα που έβαλες στο φαγητό σου (στη σαλάτα, στο ψωμί, στα ζυμαρικά ή αλλού) σε κουταλάκια του γλυκού ή της σούπας. Μην ξεχάσεις να σημειώσεις το είδος του λαδιού ή του βουτύρου (π.χ. *ελαιόλαδο, αγελαδινό βούτυρο, βιτάμ, κλπ.*).

Σύνθετα φαγητά: Για τα σύνθετα φαγητά (π.χ. *παστίτσιο, γεμιστά, σπανακόπιτα, σπανακόρυζο*), υπολόγισε την ποσότητα σε μερίδες, κομμάτια (μέτρια κομμάτια) ή κουταλιές της σούπας. Όπου είναι δυνατό δώσε πληροφορίες χωριστά για τα επιμέρους συστατικά τους.

Παραδείγματα:	*1 (μέτρια) μερίδα μουσακά* *2 κομμάτια παστίτσιο*
αλλά αντί για:	*κοτόπουλο με πατάτες στο φούρνο*
γράψε:	*1 μεσαίο μπούτι κοτόπουλου στο φούρνο και 5 πατάτες (κομμάτια μεσαίου μεγέθους) φούρνου*

ΣΤΗΝ ΠΙΣΩ ΣΕΛΙΔΑ ΣΟΥ ΔΙΝΟΥΜΕ ΕΝΑ ΠΑΡΑΔΕΙΓΜΑ
ΓΙΑ ΤΟ ΠΩΣ ΠΡΕΠΕΙ ΝΑ ΚΑΤΑΓΡΑΨΕΙΣ
ΤΑ ΤΡΟΦΙΜΑ ΜΙΑΣ ΜΕΡΑΣ.

Attachment 3.Physical activity questionnaire

ΕρωτηματολόγιοΦυσικήςΔραστηριότητας **Κωδικός**

Παρακαλούμε σκεφτείτε **<u>τις τελευταίες 7 μέρες (εβδομάδα)</u>**. Θα θέλαμε να μας δώσετε κάποιες πληροφορίες για την φυσική σας δραστηριότητα.

- Ποια είναι η βασική σας απασχόληση; _____
- Εργαστήκατε τις τελευταίες 7 μέρες;

  Όχι ☐ **➔προχωρήστε στηνενότητα 2**

  Ναι ☐ Πόσες μέρες; _____ (*1*)

  – Πόσες ώρες τη μέρα κατά μέσο όρο; _____ώρες/ ημέρα εργασίας(2)
  – Εκ των οποίων πόσο χρόνο κατά μέσο όρο καταναλώσατε:

	Ώρες/ ημέρα εργασίας	
καθιστή/ος		(*3*)
όρθια/ος		(*4*)
σε κίνηση		(*5*)
μεταφέροντας βάρος		(*6*)
Συνολικός χρόνος εργασίας		

  – Πόσος χρόνος χρειάστηκε για τη μετακίνηση σας **από και προς** τη δουλειά σας αυτές τις μέρες; _____ λεπτά/ ημέρα (*7*)
  – **Εκ του οποίου χρόνου** πόση ώρα α)περπατήσατε; _____ λεπτά/ ημέρα που πήγα στη δουλειά(*8*)
    β)οδηγήσατε; λεπτά/ ημέρα που πήγα στη δουλειά(*9*)

ΕΝΟΤΗΤΑ 1: *ΦΥΣΙΚΗ* ΔΡΑΣΤΗΡΙΟΤΗΤΑ ΣΤΗΝ ΕΡΓΑΣΙΑ
ΕΝΟΤΗΤΑ 2: ΦΥΣΙΚΗ ΔΡΑΣΤΗΡΙΟΤΗΤΑ ΣΤΟ ΣΠΙΤΙ

- Κατά τη διάρκεια των τελευταίων 7 ημερών πόσες ώρες (κατά μέσο όρο) **την <u>ημέρα</u>:**
  – κοιμηθήκατε (συμπεριλαμβανομένου και τυχόν μεσημεριανού ύπνου); _____ώρες/ ημέρα *(10)*
  – είδατε τηλεόραση-βίντεο; _____ ώρες/ ημέρα (*11*)

- Κατά τη διάρκεια των τελευταίων 7 ημερών πόσες ώρες **<u>συνολικά</u>** καταναλώσατε:
  – για ελαφριές δουλειές σπιτιού (π.χ. μαγείρεμα, πλύσιμο πιάτων κλπ); _____ώρες/ εβδομάδα (*12*)
  – για βαριές δουλειές σπιτιού(π.χ. πλύσιμο στο χέρι, σφουγγάρισμα κλπ); _____ώρες/ εβδομάδα(*13*)
  – για διάβασμα και στον υπολογιστή (εκτός ωρών εργασίας); _____ ώρες/ εβδομάδα (*14*)

- Τις τελευταίες 7 μέρες πόσες ώρες **συνολικά**:

	Ώρες/ εβδομάδα	
χορέψατε σε club ή/και bar:		(*15*)
ήσασταν καθιστός/η ή στεκόσασταν όρθιος/α με φίλους σε καφετέρια – μπαρ – ταβέρνα – εστιατόριο- θέατρο-σινεμά;		(*16*)
περπατήσατε για ψυχαγωγία (βόλτα στα μαγαζιά, στο πάρκο κλπ) και για μετακίνηση (εκτός μετακίνησης προς και από τη δουλειά):		(*17*)

- Τις τελευταίες 7 μέρες γυμναστήκατε;

  Ναι ☐ Όχι ☐

- Αν ναι τι ακριβώς κάνετε και πόσες ώρες **συνολικά** τις τελευταίες 7 μέρες:

	Ώρες/ εβδομάδα	
		(*18*)
		(*19*)
		(*20*)

ΕΝΟΤΗΤΑ 3: ΦΥΣΙΚΗ ΔΡΑΣΤΗΡΙΟΤΗΤΑ ΓΙΑ ΨΥΧΑΓΩΓΙΑ

- Με τι μέσο μετακινηθήκατε κυρίως την τελευταία εβδομάδα (σημειώστε **μόνο ένα**);

  Μοτοσικλέτα ☐ Ι.Χ. ☐ Περπατώντας ☐ Ποδήλατο ☐

  Μέσα Μαζικής Μεταφοράς (πχ. λεωφορείο, μετρό κλπ) ☐ Ταξί ☐

Τα στοιχεία θα χρησιμοποιηθούν ανώνυμα και εμπιστευτικά. ***LIMS 3***

Attachment 4.Physical and mental health questionnaire.

SF-36 ΕΡΕΥΝΑΥΓΕΙΑΣ

ΟΔΗΓΙΕΣ: Το ερωτηματολόγιο αυτό ζητά τις δικές σας απόψεις για την υγεία σας. Οι πληροφορίες σας θα μας βοηθήσουν να εξακριβώσουμε πώς αισθάνεστε από πλευράς υγείας και πόσο καλά μπορείτε να ασχοληθείτε με τις συνηθισμένες δραστηριότητές σας.

Απαντήστε στις ερωτήσεις, βαθμολογώντας κάθε απάντηση με τον τρόπο που σας δείχνουμε. Αν δεν είστε απόλυτα βέβαιος/βέβαιη για την απάντησή σας, παρακαλούμε να δώσετε την απάντηση που νομίζετε ότι ταιριάζει καλύτερα στην περίπτωσήσας.

1. Γενικά, θα λέγατε ότι ηυγεία σας είναι:

(βάλτε έναν κύκλο)

Εξαιρετική 1

Πολύ καλή 2

Καλή 3

Μέτρια 4

Κακή 5

2. Σε σύγκριση με μία εβδομάδα πριν, πώς θα αξιολογούσατε την υγεία σας τώρα;

(βάλτεένανκύκλο)

Πολύ καλύτερη τώρα απ'ότι μία βδομάδα πριν 1

Κάπως καλύτερη τώρα απ'ότι μία βδομάδα πριν 2

Περίπου η ίδια όπως μία βδομάδα πριν 3

Κάπως χειρότερη τώρα απ'ότι μία βδομάδα πριν 4

Πολύ χειρότερη τώρα απ'ότι μία βδομάδα πριν 5

1

3. Οι παρακάτω προτάσεις περιέχουν δραστηριότητες που πιθανώς να κάνετε κατά τη διάρκεια μιας συνηθισμένης ημέρας. Η τωρινή κατάσταση της υγείας σας, σας περιορίζει σε αυτές τις δραστηριότητες; Εάν ναι, πόσο;

(κυκλώστε έναν αριθμό σε κάθε σειρά)

ΔΡΑΣΤΗΡΙΟΤΗΤΕΣ	**Ναι, με περιορίζει Πολύ**	**Ναι, με περιορίζει Λίγο**	**Οχι, δεν με περιορίζει Καθόλου**
α. **Σε κουραστικές δραστηριότητες**, όπως το τρέξιμο, το σήκωμα βαριών αντικειμένων, η συμμετοχή σε δυναμικά σπόρ	1	2	3
β. **Σε μέτριας έντασης δραστηριότητες,** όπως ημετακίνηση ενός τραπεζιού, το σπρώξιμο μιας ηλεκτρικής σκούπας, ο περίπατος στην εξοχή ή όταν παίζετε ρακέτες στην παραλία	1	2	3
γ. Οταν σηκώνετε ή μεταφέρετε ψώνια από την αγορά	1	2	3
δ. Οταν ανεβαίνετε **μερικές** σκάλες	1	2	3
ε. Οταν ανεβαίνετε **μία** σκάλα	1	2	3
στ. Στο λύγισμα του σώματος, στο γονάτισμα ή στο σκύψιμο	1	2	3
ζ. Οταν περπατάτε περίπου **ένα χιλιόμετρο**	1	2	3
η. Οταν περπατάτε απόσταση **μερικές εκατοντάδες μέτρα**	1	2	3
θ. Οταν περπατάτε από σταση περίπου **εκατόμετρα**	1	2	3
ι. Οταν κάνετε μπάνιο ή όταν ντύνεστε	1	2	3

4. Την τελευταία βδομάδα, σας παρουσιάστηκαν είτε στη δουλειά σας – είτε σε κάποια άλλη συνηθισμένη καθημερινή σας δραστηριότητα – κάποια από τα παρακάτω προβλήματα, εξαιτίας της κατάστασης της σωματικής σαςυ γείας;

(κυκλώστε έναν αριθμό σε κάθε σειρά)

	ΝΑΙ	**ΟΧΙ**
α. Μειώσατε **το χρόνο** που συνήθως ξοδεύετε στη δουλειά ή σε άλλες δραστηριότητες	1	2
β. **Επιτελέσατε λιγότερα** από όσα θα θέλατε	1	2
γ. Περιορίσατε **τα είδη** της δουλειάς ή τα είδη άλλων δραστηριοτήτων σας	1	2
δ. **Δυσκολευτήκατε** να εκτελέσετε τη δουλειά ή άλλες δραστηριότητές σας (για παράδειγμα, καταβάλατε μεγαλύτερη προσπάθεια)	1	2

5. Την τελευταία βδομάδα, σας παρουσιάστηκαν – είτε στη δουλειά σας είτε σε κάποια άλλη συνηθισμένη καθημερινή δραστηριότητα – κάποια από τα παρακάτω προβλήματα εξαιτίας οποιουδήποτε συναισθηματικού προβλήματος (λ.χ., επειδή νιώσατε μελαγχολία ή άγχος);

(κυκλώστε έναν αριθμόσε κάθε σειρά)

	ΝΑΙ	**ΟΧΙ**
α. Μειώσατε **το χρόνο** που συνήθως ξοδεύετε στη δουλειά ή σε άλλες δραστηριότητες	1	2
β. **Επιτελέσατε λιγότερα** από όσα θαθέλατε	1	2
γ. Κάνατε τη δουλειά ή και άλλες δραστηριότητες λιγότερο προσεκτικά απ' ότι συνήθως	1	2

6. Την τελευταία βδομάδα, σε ποιο βαθμό επηρέασε η κατάσταση της σωματικής σας υγείας ή κάποια συναισθηματικά προβλήματα τις συνηθισμένες κοινωνικές σας δραστηριότητες με την οικογένεια, τους φίλους, τους γείτονές σας ή με άλλες κοινωνικές ομάδες;

(βάλτε έναν κύκλο)

Καθόλου 1

Ελάχιστα 2

Μέτρια 3

Αρκετά 4

Πάρα πολύ 5

7. Πόσο σωματικό πόνο νιώσατε την τελευταία βδομάδα;

(βάλτε έναν κύκλο)

Καθόλου 1

Πολύήπιο 2

Ηπιο 3

Μέτριο 4

Εντονο 5

Πολύέντονο 6

8. Την τελευταία βδομάδα, πόσο επηρέασε ο πόνος τη συνηθισμένη εργασία σας (τόσο την εργασία έξω από το σπίτι όσο και μέσα σε αυτό);

(βάλτε έναν κύκλο)

Καθόλου 1

Λίγο 2

Μέτρια 3

Αρκετά 4

Πάρα πολύ 5

9. Οι παρακάτω ερωτήσεις αναφέρονται στο πώς αισθανόσαστε και στο πώς ήταν γενικά η διάθεσή σας την τελευταία βδομάδα. Για κάθε ερώτηση, παρακαλείστε να δώσετε εκείνη την απάντηση που πλησιάζει περισσότερο σε ό,τι αισθανθήκατε. Την τελευταία βδομάδα, για πόσο χρονικό διάστημα-

(κυκλώστε ένα αριθμόσε κάθε σειρά)

	Συνεχώς	Το μεγα-λύτερο διάστημα	Σημαν-τικό διάστημα	Μερικές φορές	Μικρό διά-στημα	Καθόλου
α. Αισθανόσαστε γεμάτος/ γεμάτη ζωντάνια;	1	2	3	4	5	6
β. Είχατε πολύ εκνευρισμό;	1	2	3	4	5	6
γ. Αισθανόσαστε τόσο πολύ πεσμένος/πεσμένη ψυχολογικά, που τίποτε δεν μπορούσε να σας φτιάξει το κέφι;	1	2	3	4	5	6
δ. Αισθανόσαστε ηρεμία και γαλήνη;	1	2	3	4	5	6
ε. Είχατε πολλή ενεργητικότητα;	1	2	3	4	5	6
στ. Αισθανόσαστε απελπισία και μελαγχολία;	1	2	3	4	5	6
ζ. Αισθανόσαστε εξάντληση;	1	2	3	4	5	6
η. Ησαστε ευτυχισμένος/ ευτυχισμένη;	1	2	3	4	5	6
θ. Αισθανόσαστε κούραση;	1	2	3	4	5	6

10. Την τελευταία βδομάδα, για πόσο χρονικό διάστημα επηρέασαν τις κοινωνικές σας δραστηριότητες (π.χ. επισκέψεις σε φίλους, συγγενείς, κλπ.) η κατάσταση της σωματικής σας υγείας ή κάποια συναισθηματικά προβλήματα;

(βάλτε έναν κύκλο)

Συνεχώς 1

Το μεγαλύτερο διάστημα 2

Μερικές φορές 3

Μικρό διάστημα 4

Καθόλου 5

11. Πόσο ΑΛΗΘΙΝΕΣ ή ΨΕΥΔΕΙΣ είναι οι παρακάτω προτάσεις στη δική σας περίπτωση;

(κυκλώστε ένα αριθμό σε κάθε σειρά)

	Εντελώς Αλήθεια	**Μάλλον Αλήθεια**	**Δεν ξέρω**	**Μάλλον Ψέμα**	**Εντελώς Ψέμα**
α. Μου φαίνεται ότι αρρωσταίνω λίγο ευκολότερα από άλλους ανθρώπους	1	2	3	4	5
β. Είμαι τόσο υγιής όσο όλοι οι γνωστοί μου	1	2	3	4	5
γ. Περιμένω ότι η υγεία μου θα χειροτερεύσει	1	2	3	4	5
δ. Η υγεία μου είναι εξαιρετική	1	2	3	4	5

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