

**Report from the Probiotics, Prebiotics and Cancer Prevention
Working Group
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1. Justification

In most Western countries, cancer is the second most common cause of death and is rapidly overtaking CVD as the prime cause of mortality in many countries. Mortality from colorectal cancer (CRC) is second only to that of lung cancer in men and breast cancer in women and has shown little sign of decreasing in the last 20-30 years. Diet makes an important contribution to CRC risk, (up to 75% of CRC cases are thought to be associated with diet) – this implies that risks of CRC are potentially reducible. Evidence from a wide range of sources supports the view that colonic microflora is involved in the aetiology of CRC, which has led to intense interest in factors such as pro- and pre- biotics that can modulate gut microflora and its metabolism. In addition, the potential exists for protective effects on other human cancers, particularly breast.

2. State of the science

Evidence for protective effects of pro- and pre- biotics on cancer is derived from *in vitro* studies, animal models, epidemiology and human intervention studies. Overall, the supportive evidence is stronger for probiotics than prebiotics (possibly because the latter have only recently come to prominence) and is suggestive that synbiotics are more effective than either pro- or pre- biotics alone. The evidence from animal studies provides strongest support for anti-cancer effects and data from human studies (epidemiology and experimental) are limited.

2.1 *In vitro* studies on genotoxicity The majority of the *in vitro* studies have focussed on inhibition of chemically-induced mutation in the Ames *Salmonella typhimurium* mutagenicity test. A wide range of mutagens have been used including heterocyclic aromatic amines from cooked foods, 2-aminofluorene, aflatoxin B1 and benzo(a)pyrene. Inhibition of mutagenicity was mutagen-dependent and probiotic-species dependent. Mechanism of inhibition of mutagenicity appears to be via binding of mutagens to bacteria. This binding occurs also with non-viable cells, probably to cell wall peptidoglycans and polysaccharides and is pH dependent and reversible. In general these studies have been informative regarding potential mechanisms, but usefulness of further studies of this type are limited due the difficulties in extrapolating results especially with probiotics to the human situation.

Despite the limitations of *in vitro* studies, it was suggested that it would be of value to extend studies by:

- a) Using mammalian cell lines as targets rather than *Salmonella*.
- b) Studying endpoints other than mutagenicity (e.g. oxidative DNA damage, apoptosis, cell proliferation, COX-2).
- c) Comparing different species and strains.
- d) Exploring different mechanisms to inform hypothesis based intervention studies.
- e) Investigating effects of pro/prebiotics on gene expression and post-transcription events in colon cell lines using microarrays and 2D gel electrophoresis, which may provide insight into possible new biomarkers for cancer risk.

2.2 In vivo studies in laboratory animals (see Table 2-4) Animal studies have encompassed a large number and wide variety of animal models (conventional and transgenic rodents), inducing agents (1,2 dimethylhydrazine, azoxymethane, 2-amino-3-methyl-3H-imidazo(4,5-f)quinoline [IQ]), endpoints (genotoxicity, cell proliferation/apoptosis, putative precancerous lesions and tumours). The results show remarkable consistency, the main findings being:

- a) Anti-genotoxicity in colon: Using the comet assay, a wide range of lactobacilli and bifidobacteria, but not *Streptococcus thermophilus* has been shown to inhibit carcinogen induced DNA damage in rat colon. Inhibition was shown to be species and dose dependent, and to require viable cells. One prebiotic (lactulose) has been tested and anti-genotoxicity demonstrated.
- b) Suppression of DMH/AOM-induced aberrant crypt foci (ACF) in rat colon. Inhibition of 20-50% of ACF incidence is seen with *B. longum* and with *L. acidophilus*. More variable results with prebiotics (FOS and inulin), although synbiotic combinations were more effective than pro- and prebiotic alone.
- c) Suppression of DMH/AOM/IQ induced colon tumours. *L. casei*, *L. acidophilus* and *B. longum* have been shown to inhibit tumour incidence and *L. acidophilus* to suppress malignancy. Suppression of tumours in mutant (Min) mice and inhibition of implanted tumour growth has also been reported for FOS. There is evidence from some studies that basal diet influences pro and prebiotic outcome, with inhibitory effects being more apparent in high fat (>15% w/w) diets.

It should be noted that the relevance for humans of the DMH/AOM-induced rat colon cancer model has been questioned, as has the usefulness of the Min mouse for studying events in the colon. It is also possible that differences in gut microflora composition between rodents and humans may confound the results.

The gaps in the knowledge base where animal studies could provide further insight were considered to be:

- a) Dose response relationship, is it linear?
- b) Timing of carcinogen dose vs. pro/pre treatment, i.e. at what stage are the protective effects being exerted?
- c) Use of mouse models other than Min mouse (eg p53 knockout, MSH2 knockout).
- d) Other specific rodent models (e.g. lactobacillus-free, genetically-obese, immunocompromized, human flora associated)
- e) Elucidation of the influence of other dietary components on protective effects (e.g. protein, calcium, phosphorus, carbohydrate source).
- f) Effects of pro- and pre- biotics on other cancers (e.g. liver, mammary, prostate, bladder).
- g) Use of genomics/proteomics to investigate mechanism and potential markers for intervention studies.

2.3 Epidemiology studies (see Table 5) Epidemiological studies are key elements in the demonstration of reduction in cancer risk in humans. They provide information on a large number of people, with a wide range of ingestion of products in different cultural environments. A limited number of epidemiological studies have been conducted in different countries, on colorectal and breast cancers vs fermented milks/yoghurt consumption. Case control studies (3) indicate protective effects against colon adenomas or carcinomas, although prospective studies (3) have resulted in no significant effects. There are 2 case control studies on breast cancer both showing reduction of risk associated with fermented milk consumption.

As with all epidemiological studies, the results show associations rather than causal relationships. Furthermore, fermented milks are not precisely described in the

reported studies, so effects cannot be ascribed to specific species/strains of lactic acid bacteria

It is recommended that future studies in this area should incorporate the following important points:

- a) More careful characterization of dietary intakes to take into account possible confounding effects of other dietary components.
- b) More exploitation of epidemiological data by mining the existing sets of dietary epidemiological studies
- c) Prospective cohort studies including the simultaneous analysis of intermediate markers for insights into mechanisms and validation
- d) Comparison of the genetic background of people sensitive to or resistant to the protective effect of probiotics against cancer, might provide new understanding about mechanisms of action.
- e) Consideration of other disease entities that might be related to cancer eg insulin resistance, metabolic syndrome X, infection, colitis.

2.4 Human experimental/intervention studies (see Table 6). To date 10 human intervention studies with lactic acid producing bacteria (LAB) or yoghurts have been reported. The majority have been performed in healthy volunteers, some in polyp/cancer patients, and have employed faecal bacterial enzymes, faecal steroids, faecal flora as endpoints. Mostly lactobacilli have been studied, and there are some studies on FOS. Although such studies are technically more difficult than animal studies, they do provide information of direct relevance to the human situation, indicate causal relationships and give insight into potential protective mechanisms in human. In general, the changes in studied biomarkers to date have suggested a reduction in risk. Few data on dose response relationships have been reported

Data from human intervention studies are of paramount importance in providing evidence that LAB, prebiotics or fermented milk consumption are causally related to reduction in cancer risk. Thus, this is an area of high priority for future studies. Presently however the lack of well validated biomarkers limits the relevance of such studies although a wide range of potential biomarkers of risk are under development (see below). Once such markers are available, it will become possible to perform studies in healthy volunteers, at-risk groups and patients. It will be important to define dose and time relationships and it would appear, from animal studies, the most profitable approach would be to use combinations of pro- and pre-biotics. There will also be, in the near future, the opportunity to exploit genomics and proteomics in investigations of effects of pro/prebiotics on gene expression and post-transcription events in colonic biopsies and to identify human groups responsive to pre/probiotic intervention. It will be particularly important to use data on mechanisms of action to develop hypothesis based intervention studies in humans

3. Potential mechanisms of anti-carcinogenicity and anti-genotoxicity

3.1. Binding/adsorption of carcinogens: There are several reports of adsorption or binding of carcinogens, such as heterocyclic amines (IQ, MeIQ, PhIP, Trp-P-2, Glu-P-1), Aflatoxin B1 and benzo(a)pyrene, *in vitro* by LAB and other intestinal bacteria. A concomitant decrease in mutagenicity is often reported. The extent of binding is dependent on the mutagen and bacterial strain, and binding was shown to be a physical phenomenon, not requiring viable cells. There is conflicting evidence as to *in vivo* relevance.

3.2. Effects on bacterial enzymes, metabolite production: Administration of some LAB/fermented milks and/or prebiotics to animals and humans leads to decreases in certain bacterial metabolites and enzymes purported to be involved in synthesis or

activation of carcinogens, genotoxins and tumour promoters such as β -glucuronidase, β -glucosidase, nitrate reductase, ammonia. Some studies have shown changes in enzyme activities concomitant with suppression of tumours or ACF. Although a causal link has not been demonstrated, this remains a plausible hypothesis.

3.3. Stimulation of host enzymes involved in carcinogen inactivation : There are isolated reports demonstrating that LAB and/or prebiotic administration to rats results in increased activity of enzymes or processes that protect cells against carcinogen induced damage. These include glutathione transferase (induced by *B. longum* and lactulose and resistant starch), hepatic uridine diphosphoglucuronyl transferase, colonic NADPH-cytochrome P450 reductase (induced by various LAB) and enhanced removal of O⁶-methylguanine from colonic mucosa in AOM treated rats given LAB. Such mechanisms of protection could be effective against a wide range of dietary carcinogens possibly influencing several cancer sites.

3.4 Alteration of apoptosis and cell proliferation: Administration of inulin/FOS to AOM treated rats increased apoptosis in colon, especially distal colon (1 study) and LAB inhibited AOM-induced cell hyperproliferation and ornithine decarboxylase activity in rats (1 study).

3.5. Modulation of immune/inflammatory response: Suppression of colon tumour growth by yoghurt in DMH treated mice was associated with suppression of the inflammatory immune response (Perdigon *et al* 1998). An immune mechanism has been proposed (without supporting evidence) to explain the increase in time before tumour recurrence in bladder cancer patients given *L. casei*.

3.6. Other potential mechanisms: Several additional potential mechanisms have been proposed, usually with limited data to support them. These include: colonic fermentation products (eg butyrate, lactate), bioactive components (eg peptides, nisin, bacteriocins) produced in fermented milks, increased mucus production or changes in the mucus profile; calcium activity on epithelial mucosa and in lumen; decrease in gut transit time

4. Human intervention studies

Data from human intervention studies is crucial for confirming the positive effects seen on cancer in animal studies. The type of subject to be used is an important factor when designing these studies. Studies in healthy subjects limits sampling to faeces, blood and urine, whereas with adenoma or even cancer patients, biopsies are possible, so tissue markers can be used, or adenoma growth and recurrence can be assessed.

At risk groups, such as FAP, HNPCC, include useful subjects, but as with all patients the question of relevance of the results to the normal population arises.

Study designs should be randomized, double blind, with control, and the length of time on the study needs to be appropriate for markers employed. It is extremely important to collect information on background diet being consumed.

An large range of biomarkers is available for use in human intervention studies and may be divided into 4 main groups as follows:

1. Cancer/adenoma endpoints: Cancer incidence (true endpoint but impractical), adenoma recurrence, Adenoma growth, colonic ACF (in development).

2. Tissue markers (assessed in biopsies) Mucosal cell proliferation, apoptosis, DNA adducts, DNA damage, DNA repair/ Mismatch repair, oncogene/suppressor gene mutations, Cox-2 gene expression, GST activity/expression, cytochrome 450 activity, sialomucins/sulphomucins, genomics (microarrays, RT PCR), proteomics.

3. Faecal markers - Biochemical: enzymes (glucuronidase etc), ammonia, N-nitrosocompounds, DAG, secondary bile acids, Calprotectin

4. Faecal markers - Faecal water, Cytotoxicity, genotoxicity, apoptosis, AP1 gene transcription, COX-2 induction.

In addition to the above, it is possible to sample surrogate sites for endpoints that might provide information on cancer risk at colon and other sites in the body For example, in breath: profile of volatile organic compounds (VOCS); in blood: antioxidant levels, oxidative DNA damage, homocysteine/folate, C-reactive protein, IGF and binding proteins; in urine: p-cresol and phenols, oxidative DNA damage (O6-deoxyguanosine); buccal mucosa: genomics and proteomics

It is important to note that most of the above markers of cancer risk have not been fully validated, which seriously limits the conclusions that can be drawn from human intervention studies in relation to cancer risk reduction . Such validation should be a prime focus of research activities.

5. Summary of main issues that need to be addressed

- Identification of main mechanisms of action. (*in vitro* and animal studies)
 - Do pro and prebiotics influence similar and/or synergistic mechanisms?
 - Do different species & strains influence similar mechanisms ?
 - What are the criteria for efficacy (viability? Survival and activity in GI tract?, Delivery system?
 - Are all prebiotics similar?
- Dose response and time effects - Critical period of consumption of pro/prebiotics for optimal effect on cancer (animal studies)
- What markers can be recommended for epidemiology studies?
- Validation of biomarkers for intervention studies.
- Are there subpopulations of humans with specific sensitivity/resistance to pro and prebiotics?
- Corroboration of results from animal models in humans.
 - Epidemiology studies with better dietary characterization and surrogate markers of risk.
 - Intervention studies with validated biomarkers.

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