Dietary fibre supplementation enhances radiotherapy tumour control and alleviates intestinal radiation toxicity

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Abstract

Background

Non-toxic approaches to enhance radiotherapy outcomes are beneficial, particularly in ageing populations. We investigated the efficacy of high-fibre diets combined with irradiation in C57BL/6 mice bearing bladder cancer flank allografts.

Result

Psyllium plus inulin significantly decreased tumour size and delayed tumour growth following irradiation compared to 0.2% cellulose and raised intratumoural CD8\(^+\) cells. Post-irradiation, tumour control positively correlated with Lachnospiraceae family abundance. Psyllium plus resistant starch radiosensitised the tumours, positively correlating with \textit{Bacteroides} genus abundance. Although local immunity was suppressed, psyllium plus resistant starch increased caecal isoferulic acid levels, associated with a favourable response. Both diets mitigated the acute radiation injury caused by 14 Gy. All high fibre diets increased caecal short-chain fatty acid levels. Similar gut microbiota profiles in cancer patients and low-fibre diet mice indicates scope for testing dietary fibre supplements in human intervention studies.

Conclusion

These supplements may be useful in combinations with radiotherapy in patients with pelvic malignancy.

Introduction

Radiation +/- concurrent chemotherapy has an important role in the treatment of pelvic malignancies, such as bladder, prostate and colorectal cancers, as it allows organ preservation[1, 2]. However, elderly people, who are more vulnerable to treatment-related toxicity, make up the majority of patients with pelvic cancers. Interventions that can enhance response, and organ preservation, without additional toxicity are needed urgently.

The gut microbiota has a significant, potentially beneficial, impact on human health and disease[3, 4]. Its composition was associated with response to immunotherapy in cancer patients and causality has been demonstrated preclinically[5, 6]. Gopalakrishnan \textit{et al.} showed that melanoma patients responding to anti-programmed cell death 1 protein (PD-1) immunotherapy had significantly higher alpha diversity (a measure of taxonomic diversity within communities) of the gut microbiota and Ruminococcaceae family abundance[5]. Studies also found a similar association between higher alpha diversity and chemoradiation response in cervical cancer patients[7] and in colorectal patients[8]. Preclinical mouse models also support the hypothesis that the gut microbiota can modulate the efficacy of chemotherapy[9,
and radiotherapy[11, 12]. The enhancement of anti-cancer treatment can be achieved via immunomodulation and/or secretion of metabolites, including butyrate[13, 14], inosine[15] and trimethylamine-N-oxide (TMAO)[16]. Dietary fibre is fermented by the gut microbiota to produce short-chain fatty acids (SCFAs) and a broad range of other metabolites[17].

An effective way to modify the gut microbiota is by adding dietary fibre supplements to the diet that can alter human gut bacterial diversity and faecal SCFA production rapidly[18]. Mounting preclinical evidence shows that dietary fibre can slow tumour growth[19, 20] and enhance the efficacy of anti-cancer treatments[21]. Spencer et al. showed that melanoma patients with high dietary fibre consumption had a better response to immunotherapy[22], and this had a protective effect against gastrointestinal toxicity during pelvic radiotherapy in a randomised-controlled clinical trial[23].

To our knowledge, there are no human studies of dietary fibre supplements in the context of enhancing tumour response to pelvic radiotherapy. We were the first to use dietary fibre to enhance radiosensitivity in an immunocompromised mouse bladder tumour model[24] and showed that this was associated with modification of the gut microbiota. The main alternative strategy in vivo has used antibiotics in studying the role of the gut microbiota in relation to radiotherapy efficacy[11, 25]. These preclinical cancer models showed that antibiotics can diminish[25] or increase[11] the radiotherapy effect with depletion of specific commensal bacteria.

In this study, we used immunocompetent C57BL/6 mice to study the effects of dietary fibre supplementation, alone and in combination with ionising radiation (IR), on tumour response and radiation normal tissue toxicity. We showed that the systemic effects of the gut microbiota in terms of secreted metabolites and immune responses, due to dietary fibre modification, could be exploited in conjunction with radiotherapy to achieve tumour radiosensitisation and amelioration of normal tissue effects.

**Results**

*Psyllium plus resistant starch (RS) or psyllium plus inulin significantly decreased tumour size*

UPPL1591 mouse bladder tumour cells were inoculated subcutaneously on the same day as starting to feed the mice with normal chow or a modified diet, namely, 0.2% cellulose, psyllium (used to mitigate side effects after radiotherapy), psyllium plus resistant starch (RS; butyrate-producing fibre) or psyllium plus inulin (readily fermentable fibre and potential radiosensitiser[24]). Psyllium plus either RS (p = 0.007) or inulin (p < 0.001) significantly delayed tumour growth compared to the 0.2% cellulose group (Fig. 1a). The mean tumour sizes were 80 mm$^3$ and 60 mm$^3$ in psyllium plus RS and psyllium plus inulin, respectively, at the time that the 0.2% cellulose and psyllium groups reached 100 mm$^3$. With time to reach 100 mm$^3$ as the survival analysis endpoint, log-rank testing showed median times were significantly different (p = 0.037) among dietary groups, at 15, 13, 13, 16 and 21 days for normal chow, 0.2% cellulose, psyllium, psyllium plus RS and psyllium plus inulin, respectively (Fig. 1b).
The gut microbiota profiles showed that the three taxa with the highest abundances were a *Muribaculaceae* uncultured species, *Lactobacillus* species and *Bifidobacterium animalis* (Fig. 1c). Psyllium plus inulin increased the *B. animalis* abundance up to 35% which was significantly higher than in all the other dietary groups (Fig. 1d). *Bacteroides* genus was significantly enriched in mice fed the psyllium diet (Supplementary Fig. 1). Mice fed with psyllium plus RS had higher abundances of *Parasuterella* (the top genus enriched by this diet) and *Faecalibaculum* (butyrate-producing bacteria that possess anti-tumorigenic properties[26]) genus (Supplementary Fig. 2a) and the alpha diversity was slightly higher than that of psyllium group (p = NS; Supplementary Fig. 2b). A notable cluster effect of all dietary groups was found (Fig. 1e).

It is acknowledged that the cytotoxic T cell is the most powerful effector cell in anti-tumour immunity[27]. For all tumour samples, the number of CD8\(^+\) T cells was significantly increased by psyllium plus inulin compared to psyllium plus RS (p = 0.012; Fig. 1f). Immune cell profile by a NanoString platform also showed a consistent result with the ratio of CD8\(^+\) T cells to total T cells enriched in psyllium plus inulin group tumours (p = 0.049; Fig. 1g), this being the only immune cell type increased (Supplementary Fig. 3a). Psyllium plus inulin-treated mice also tended to have higher levels of systemic helper T and cytotoxic T cells compared to psyllium plus RS-treated (p = NS; Supplementary Fig. 3b), consistent with the local tumour immune analysis. Metabolite profiles were also modified by the dietary fibre with differences between psyllium plus RS, psyllium plus inulin and psyllium alone (Supplementary Fig. 4a) and inosine was significantly enriched in the caecal contents of mice fed with psyllium plus inulin along with N-acetyl ornithine, N6-acetyl-L-lysine and homocitric acid (Supplementary Fig. 4b). Sumiki’s acid, 4-(2-aminophenyl)-2,4-dioxobutanoic acid, allantoin and cysteine-S-sulfate were enriched in mice fed with psyllium plus RS.

**Psyllium plus RS or psyllium plus inulin combined with IR increased growth delay in bladder tumours**

To study the radiosensitising effect of dietary fibre, we irradiated (IR) the tumours with 6 Gy IR when they reached 80–100 mm\(^3\), with all other parameters as per the previous diet alone UPPL1591 tumour experiment (Fig. 2a). This experiment consisted of 60 mice in four groups (n = 15 per group) fed modified diets either without (n = 5) or with (n = 10) irradiation. Across the whole experiment, all mice stably gained weight in the low and high fibre diets groups (Fig. 2b). All psyllium diets maintained the rate of weight increase, especially psyllium plus RS (p = 0.047) and psyllium plus inulin (p < 0.001), compared to 0.2% cellulose. After receiving IR when the tumours reached 80–100 mm\(^3\), IR cohorts of psyllium plus RS (p = 0.033) or inulin (p < 0.001) experienced slower rates of weight gain compared to their non-IR controls (Supplementary Fig. 5).

In all dietary groups, IR slowed tumour growth compared with their non-IR controls (p-values were < 0.001 for 0.2% cellulose, psyllium plus RS or psyllium plus inulin, and 0.039 for psyllium; Fig. 2c, d). In the IR cohorts, psyllium plus RS or psyllium plus inulin significantly delayed tumour growth compared with either 0.2% cellulose (p < 0.001 and = 0.028) or psyllium (p < 0.001 and = 0.034; Fig. 2c, d). To assess the specific radiosensitising effects of the high fibre diets (i.e. removing the effect of diet alone in delaying
tumour growth), we aligned Day 0 to the day of tumours reaching 80-100mm$^3$ or receiving IR, and compared the tumour growth curves among different dietary groups (Supplementary Fig. 6a). Psyllium plus RS significantly radiosensitised bladder tumours compared to 0.2% cellulose ($p = 0.004$) and psyllium ($p < 0.001$). This result showed that psyllium plus RS had a greater effect than psyllium plus inulin in mediating tumour control by ionising radiation and the benefit seen with psyllium plus inulin was a composite effect of high dietary fibre influencing tumour growth per se and subsequent IR (Fig. 1a, 2c). We conducted further survival analysis by using the time to quadruple tumour volume as an endpoint (Supplementary Fig. 7). Irradiated mice in all dietary groups had significantly longer median times for tumours to quadruple in volume compared to non-IR controls and the p-values were 0.021 for 0.2% cellulose, 0.034 for psyllium, < 0.001 for psyllium plus RS and 0.004 for psyllium plus inulin. These data reflect 6 Gy conferring some degree of survival advantage and demonstrate a greater impact of irradiation in the group fed psyllium plus RS compared to psyllium plus inulin. To assign responders and non-responders, we split the mice into two clear groups for each diet at a tumour volume of approximately 400mm$^3$ at day 26 post IR. There were three responding mice who received 0.2% cellulose, and five mice in the psyllium, eight mice in the psyllium plus RS and four mice in the psyllium plus inulin groups (Supplementary Fig. 6b).

The gut microbiota profile shows that a *Muribaculaceae* uncultured bacterium, *Bacteroides* species and *Bifidobacterium animalis* were the three bacteria with the largest abundance among all the other bacteria taxa (Fig. 2e). These taxa were consistent with the result of the previous cohort in Fig. 1c. To explore how specific bacterial taxa affect tumour control in the psyllium plus inulin group, we investigated the correlation between Lachnospiraceae family abundance versus tumour growth rate, because this was the top enriched taxon for responders (Supplementary Fig. 8a). In this diet group, tumour growth post-inoculation was negatively correlated with Lachnospiraceae family relative abundance in the IR cohort ($p = 0.013$; Fig. 2f) but this was not seen in the non-IR cohort ($p = 0.974$; Supplementary Fig. 8b). In the psyllium plus RS group, the two non-responders were more alike than the responders to IR, unlike the psyllium plus inulin group (Supplementary Fig. 9). The *Bacteroides* genus comprised around 14 to 20% of the gut microbiota in mice in the psyllium plus RS group (Supplementary Fig. 10). Its abundance was higher in the IR cohort, and it was significantly associated with a better tumour response to irradiation ($p = 0.007$), in contrast to the non-IR cohort (Fig. 2g, Supplementary Fig. 10). *Parasuterella* and *Faecalibaculum* genera, enriched in the mice fed with psyllium plus RS (Supplementary Fig. 2a), were associated with better tumour control in the non-IR cohort of this diet group but not the IR cohort (Supplementary Fig. 10).

*Enhanced immune response and metabolite production by psyllium plus inulin or psyllium plus RS combined with IR in bladder tumours*

NanoString analysis provided an overview of the immune cell profile and immune-related gene enrichment (a set of genes whose expression is over-represented) in tumours. Consistent with the intra-tumoural immune responses in Fig. 1f, the number of CD8 cells was increased in the psyllium plus inulin group but decreased in the psyllium plus RS group after irradiation (Fig. 3a, Supplementary Fig. 11). In
addition, the other immune cells, including neutrophils and NK cells, were also slightly elevated in the psyllium plus inulin group compared to the RS group (p = NS; Supplementary Fig. 12a). Most immune pathways were enriched in tumours of the psyllium plus inulin group compared to psyllium plus RS, especially humoral immunity, cytokines and their receptors, and interferon, along with Bst1 and Nfatc2 gene expression (Fig. 3b, Supplementary Fig. 12b). The immune-related genes with higher expression levels in psyllium plus RS were Tgfb3, Nrp1, Fnn1, and Ada (Supplementary Fig. 12b). Since local tumour immune response was enhanced by psyllium plus inulin, we investigated whether this local tumour and/or systemic immunity was associated with tumour response. In tumours, the number of exhausted CD8 cells significantly decreased in responders (p = 0.043; Supplementary Fig. 13a), while the others, including NK cells, dendritic cells, NK CD56 dim cells and T cells, slightly increased (p = NS; Fig. 3c). In addition, the T cell functional pathways, and cytokines and receptors were enriched in responders to psyllium plus inulin (Supplementary Fig. 13b). For T cell function, the significantly up-regulated genes which had an adjusted p-value of < 0.01 were Bcl10, Il2ra, Il18rap, Card11, Cd5, Tnf, Dpp4, Il12b and Fasl. For cytokines and receptors in responders, there were seven genes significantly up regulated, namely, Traf3, Il18rap, Card11, F2rl, Il12b, Tnf and Fasl, while the Il1r2 gene was down regulated. This implies that the up-regulation of genes related to T cell function, and also cytokines and relevant receptors may be needed for the tumour response to IR and psyllium plus inulin. For the effector cells, tumour growth rate negatively correlated with splenic cytotoxic T cells (p = 0.095, Fig. 3d). GM-CSF and IL-2 were two cytokines belonging to Th1 cytokine panel which have been used to improve the efficacy of radiotherapy by activations of immune cells and enhancement of antigen presentations[28]. There were negative correlations between these two cytokines and tumour growth (p-values 0.048 for GM-CSF and 0.071 for IL-2, respectively; Supplementary Fig. 13c). To explore whether the gut microbiota modulate the systemic immune response, we assessed the correlation between Clostridia and Lachnospirales (bacterial taxa associated with tumour control) abundance versus systemic immune cells. There were significant correlations between the abundance of these orders and splenic cytotoxic T cells (R^2 = 0.618, p = 0.006; Fig. 3d) and macrophages (R^2 = 0.460, p = 0.030; Supplementary Fig. 14). This implies that the dietary fibre-modified gut microbiota might be required to activate systemic immune responses.

Discovery metabolomics was performed to compare the caecal metabolome of mice fed with different dietary fibres. Principal Components Analysis (PCA) showed a notable clustering effect of metabolite profiles by diet (Fig. 3e). Consistent with the gut microbiota profile in Fig. 1e, the psyllium and psyllium plus RS groups were more similar to each other compared to the other dietary groups. An ANOVA analysis comparing the metabolite levels showed that, compared to the 0.2% cellulose dietary group, the psyllium plus RS group had a significantly higher level of isoferulic acid (false discovery rate = 9.08E-9; Fig. 3f), a metabolite previously shown to inhibit human leukemic cell growth[29]. Isoferulic acid was also associated with better tumour control in the psyllium plus RS group (R^2 = 0.540, p = 0.015; Fig. 3g). The unfavourable metabolites (positively correlated with tumour growth) were related to amino acid metabolism, namely, glycine, serine, threonine, cysteine, and methionine (Supplementary Fig. 15). In the IR cohort of psyllium plus inulin, threitol was associated with improved tumour control (R^2 = 0.559, p = 0.012; Supplementary Fig. 16). Asparaginyl-hydroxyproline (R^2 = 0.495, p = 0.007) and butyrate (R^2 = 0.272, p =
levels were also associated with a slower tumour growth rate in IR cohort and non-IR cohort in the psyllium plus inulin group.

*Dietary fibre manipulation spares IR-induced normal tissue toxicities*

While aiming to improve tumour control is key, it is also advantageous in terms of the therapeutic ratio to reduce normal tissue toxicity if possible. As there is evidence of the benefits of SCFA and dietary fibre in relieving radiation-induced symptoms[30, 31], we investigated whether dietary fibre supplementation could spare acute intestinal toxicity caused by radiation (Fig. 4a). After feeding the mice with modified diets for two weeks, we irradiated their lower abdomen, centred on the urinary bladder, and covering part of the small and large intestines, with 10, 12 and 14 Gy on the Xstrahl small animal radiation research platform (SARRP) (Fig. 4b). We applied our modified crypt assay to investigate whether there was a protective effect conferred by the dietary fibre. There was no difference among the different dietary groups when the mice received 10 and 12 Gy IR, but psyllium plus RS (p = 0.087) and psyllium plus inulin (p = 0.007) increased the number of crypts remaining in mice receiving the higher 14 Gy dose of IR (Fig. 4c). Gut microbiota profiles showed a similar composition within the same dietary groups before and after irradiation (Supplementary Fig. 17) and, in terms of beta diversity (a measure of similarity between communities), PCA showed a notable cluster effect among the gut microbiota for all psyllium diets groups (Supplementary Fig. 18a). In mice receiving different doses of SARRP IR within the same dietary group, we found that the higher the dose of IR, the larger the distance of the gut microbiota from the non-IR (0 Gy) controls (Supplementary Fig. 18b). All radiation doses significantly changed the gut microbiota in 0.2% cellulose (p = 0.018, 0.003 and < 0.001), while for psyllium plus RS (p = 0.012 for 12 Gy) or psyllium plus inulin (p = 0.010 and < 0.001 for 12 and 14 Gy) only 12 Gy and 14 Gy caused significant changes. One of the major physiological functions of the gut microbiota is secretion of metabolites. The PCA of the discovery metabolomics analysis showed that psyllium and psyllium plus RS had more similar metabolomics profiles relative to psyllium plus inulin (Supplementary Fig. 18c). In addition, we saw that IR changed the metabolomic profiles in each dietary group, as the higher the IR dose, the greater the difference in metabolomic profile compared to non-IR controls (Supplementary Fig. 18d). All psyllium-containing diets significantly increased the caecal SCFA levels, but not isovaleric acid and isobutyrate, after three-weeks of modified diet (Fig. 4d, Supplementary Fig. 19a). Psyllium significantly raised the acetate (p < 0.001) and propionate (p = 0.002) levels, and psyllium plus RS increased acetate (p < 0.001 and = 0.003) and butyrate levels. Psyllium plus inulin resulted in the highest levels of acetate, propionate and butyrate among all dietary groups and the p-values were < 0.001 for all three SCFAs compared to 0.2% cellulose.

Patients may develop long-term side effects from 3 months after receiving pelvic radiotherapy. To validate whether dietary fibre and the gut microbiota can protect the intestine from late radiation-induced injury, we performed an experiment irradiating the mice with 5 x 5 Gy IR to the lower abdomen, including their urinary bladder and the lower part of the large intestine, avoiding the small intestine by treating the mice head down (Fig. 4e, f). The mice were then followed up to 24 weeks. Figure 4g shows the weight changes within 3 weeks after IR. The IR cohort in the 0.2% cellulose group experienced delayed weight gain compared to their non-IR cohorts, not seen in the psyllium diet groups. The delayed weight gain in
0.2% cellulose was significant (p = 0.006) during the first week but gradually recovered subsequently. Consistent with the acute toxicity experiment, psyllium plus RS and psyllium plus inulin had higher butyrate levels compared to 0.2% cellulose after nine-weeks of modified diet (Supplementary Fig. 19b). Five weeks after changing back to normal chow from the modified diets, minor clustering effects for all diet groups were still found and all psyllium diet groups were rather different from 0.2% cellulose (Supplementary Fig. 20, 21), but we found no significant fibrosis in the colons at 20-week post-irradiation (Supplementary Fig. 22).

**Bacterial supernatants of co-cultures of B. acidifaciens and a butyrate-producing bacterium induced greater cytotoxic response, histone deacetylase inhibition, IR-induced DNA damage and radiosensitivity**

In our previous study, *B. acidifaciens* was enriched in responders to irradiation, and we proposed this *Bacteroides* species might be a potential radiosensitiser[24]. In this current study, we also found that the *Bacteroides* genus was associated with tumour control by psyllium plus RS. So, we used bacterial supernatants to investigate the anti-tumoural properties of *Bacteroides acidifaciens* as a model organism for *Bacteroides* spp (Fig. 5a). There was very limited knowledge about this bacterium although it produces acetate[32], required for butyrate production. Therefore, we compared the cellular effects of *B. acidifaciens* to the well-known acetate producer, *Bifidobacterium animalis*. Given that HDAC inhibition is a promising mechanism of radiosensitisisation[33], the results showed that bacterial supernatants from the co-culture of *B. acidifaciens* (BA) plus *F. prausnitzii* (FP) significantly increased histone acetylation (Fig. 5b) and the cytotoxic response of RT112 (Fig. 5c) and T24 (Supplementary Fig. 23a) human bladder cancer cell lines compared to the other supernatants including *B. acidifaciens* alone, *B. animalis* (acetate-producer), *F. prausnitzii* (butyrate-producer) and the co-culture of Bif + FP. Similarly, treating RT112 and T24 cells with BA + FP supernatant demonstrated greater delay in repair of γH2AX nuclear foci by immunofluorescence microscopy than for each of the individual supernatants (Fig. 5d, Supplementary Fig. 23b). Also, four hours after 5 Gy IR, there were significantly higher levels of DNA damage in BA + FP compared to the other supernatants, except Bif + FP, as measured by γH2AX protein levels (Supplementary Fig. 24). We further studied the radiosensitising properties of bacterial supernatants of BA + FP by irradiating the bladder cancer cells from 0–8 Gy (Fig. 5e). Clonogenic assay showed that the supernatant can radiosensitise RT112 bladder cancer cells in a dose-dependent manner. The metabolite profile by bacteria showed that ADP, ribulose 1,5-diphosphate, isovalerylglucose, butyrate (p = 4.22E-05, FDR = 7.38E-04), D-N-(carboxyacetyl)alanine and carbamoyl isoleucine were enriched in the supernatants of BA + FP, that had significantly higher cytotoxic responses, compared to *B. acidifaciens* or *F. prausnitzii* alone (Fig. 5f, g). Therefore, we have demonstrated that *B. acidifaciens* is a potential radiosensitiser with enhanced efficacy in combination with butyrate-producing bacteria.

**Comparison of microbial profiles between human and mouse faecal samples**

Analysis of phylum-level gut microbiota composition showed that the cancer patients’ profiles were more similar to those of mice fed a low fibre diet (0.2% cellulose) than the other high fibre diet groups, with higher Firmicutes and lower Proteobacteria abundance (Fig. 6a, supplementary Table 1). SCFA analysis of cancer patient samples demonstrated a broad range of faecal SCFA levels among individuals,
especially acetate, propionate, and butyrate (supplementary Fig. 25a). We found that the abundance of several bacterial taxa was significantly different in patients with either high or low faecal acetate, propionate and butyrate by using the median of the three SCFAs combined as cut-off value (Fig. 6b, supplementary Table 2, supplementary Fig. 25b). We have confirmed that the effect of refrigerated storage time (up to 3 days) on stool samples was minimal. Samples from three individuals processed serially at 24-hour intervals (0, 24, 48 and 72 hours) had no significant intra-individual differences in microbial composition, diversity or SCFA concentrations (supplementary Fig. 26a-d, supplementary Table 3). Despite a significant difference in between-individual gut microbiota profiles, among all SCFAs, only butyrate was lower in sample 3 compared to samples 1 and 2 (Fig. 26d).

There were nine bacteria taxa enriched in the high SCFA group, namely, Bacteroides coprophilus, Agathobacter genus, Lactobacillus johnsonii, Agathobacter species, Parabacteroides merdae, Dorea genus, Prevotellaceae_NK3B31_group genus, Dorea uncultured bacterium species and Lactobacillus ruminis. It is noted that four of these belong to Lachnospiraceae, a family that was associated with better tumour control by psyllium plus inulin in mice (Fig. 2c, d). We also showed that Lachnospiraceae family abundance in cancer patients was positively correlated with the total concentration of three major SCFAs from gut microbiota ($R^2 = 0.056$, $p = 0.038$), acetate ($R^2 = 0.068$, $p = 0.022$), butyrate ($R^2 = 0.051$, $p = 0.049$) and valerate ($R^2 = 0.102$, $p = 0.005$) concentrations, but not formate and propionate (Fig. 6c, supplementary Fig. 27). Regarding another favourable gut bacterium (Fig. 2g), there was also a trend of association between Bacteroides genus versus faecal formate level ($p = 0.065$, supplementary Fig. 28).

Discussion

To our knowledge, we have been the first to use dietary fibre to enhance radiosensitivity in an immunocompromised bladder tumour model[24]. Here, in an immunoproficient mouse model, we have shown that psyllium plus RS and psyllium plus inulin were favourable dietary fibre combinations that significantly improved radiotherapy efficacy and also mitigated the acute intestinal side effects of radiotherapy. In cancer patients receiving radiotherapy, supplementation of several different dietary fibres can mitigate the side effects of radiotherapy. For example, psyllium has been shown to be effective in reducing the incidence and severity of radiation-induced diarrhoea[34]. RS is well-known as a source of butyrate production via bacterial fermentation in the colon[35], and butyrate is the main energy source of colonocytes and is an anti-inflammatory agent. In a large clinical study of patients receiving radiotherapy to their prostate and pelvic lymph nodes, radiotherapy changed the gut microbiota, and this was associated with early and late enteropathy in patients[36]. In a recent systematic review including twenty-three randomised, controlled trials, we also showed that biotic supplements, especially probiotics and synbiotics, reduce acute symptoms of diarrhoea in patients undergoing pelvic radiotherapy[37]. In terms of tumour control, psyllium plus inulin had the largest effect compared to 0.2% cellulose, psyllium, and psyllium plus RS. This result is supported by previous studies where inulin delayed tumour growth of mouse breast cancer[19] and enhanced tumour control combined with irradiation in mouse bladder tumours[24], thereby demonstrating anti-tumour effects of inulin in cancers outside the intestines,
and its ability to enhance the efficacy of anti-cancer treatments. We found that psyllium plus inulin increased tumour growth delay, when combined with irradiation, compared to 0.2% cellulose and psyllium. Tumour growth rate significantly negatively correlated with relative abundance of the Lachnospiraceae family, belonging to Clostridiales order, which emphasises the importance of gut microbiota modification in enhancing tumour control. A previous study, in mice receiving melanoma immunotherapy, also supports our finding that Clostridiales abundance is associated with enhancement of systemic CD8+ cells[5]. Furthermore, our discovery metabolomics analysis showed that psyllium plus inulin increased level of caecal inosine that has previously been shown to enhance immunotherapy efficacy via activation of cytotoxic T cells[15, 38].

There are very few studies regarding resistant starch and tumour growth outside colorectal cancer. Some resistant starches (whole wheat bread, legumes and boiled potato) are associated with reduced breast cancer risk[39] while Hi-Maize 260 RS decreased tumour size in a mouse pancreatic cancer model[40]. Mathers et al. also found that 30 g/day RS can reduce development of extracolonic cancers in Lynch Syndrome patients[41]. In this study, we showed that, although psyllium plus RS had a smaller effect on tumour control compared to psyllium plus inulin, it conferred a higher radiosensitising effect. The IR response in psyllium plus RS negatively correlated with Bacteroides genus, suggesting that the IR response was associated with the gut microbiota. From the discovery metabolomic analysis, isoferulic acid was significantly enriched in mice fed with psyllium plus RS and was associated with better tumour control in this diet group. Ferulic acid, an isomer of isoferulic acid, has been shown to suppress homologous recombination-dependent repair in breast cancer cells[42] and even confer a radiosensitising effect in lung[43], liver[43] and cervical[44] cancer cells. It is reported that, compared to ferulic acid, isoferulic acid had a higher inhibitory potency on a murine macrophage cell line[45]. The suppression of the immune response with a higher expression of the immunosuppressive cytokine gene Tgfb3 in psyllium plus RS again suggests that its radiosensitising effect might be achieved via non-immune mechanisms.

Consistent with the association between the Bacteroides genus and tumour control post-IR by psyllium plus RS, Bacteroides acidifaciens was identified as a potential radiosensitiser in our previous study, where its abundance was positively correlated with tumour response to irradiation [24]. Studies suggest that a broad range of gut microbiota-derived metabolites can enhance anti-tumoural effects or tumour response to anti-cancer treatments[46]. Beyond SCFAs, future work should include identification of radiosensitisers with similar profiles from the global metabolomic analysis of B. acidifaciens. In addition, greater cytotoxic phenotypes (Fig. 5b, c, d) generated by co-culture of BA and butyrate-producing bacteria also suggests the need to build a collection of combinations of bacteria, including BA and other butyrate producers, to pursue a better response than achieved with a single bacterium alone. This was shown in a previous study of a community of 11-strains, primarily rare, low-abundance components of the human microbiota, which enhanced the therapeutic efficacy of immunotherapy in a mouse colorectal cancer model[47].
Spencer et al. also showed that high dietary fibre increased the efficacy of melanoma immunotherapy in both mouse and human[22]. This phenomenon was associated with the enhancement of T cell function, also seen in this study[22]. However, this effect was diminished when combining the dietary fibre with probiotics – Lactobacillus or Bifidobacterium. This implies that dominance of these probiotic species may have overridden the gut microbiota that could have enhanced the efficacy of anti-cancer treatments. This evidence supports dietary fibre as a better approach to enhance tumour control rather than dosing with a small number of specific bacteria, because it can shape the whole gut microbiota to a more favourable profile, with more diverse metabolites produced. In addition, Hu et al. showed that beta-glucan (Maitake) mitigated chemoradiation-related adverse effects in head and neck cancer patients in a randomised clinical trial[48].

In this study, five sets of experiments using modified diets from at least two weeks up to nine weeks did not show abnormalities of appearance or behaviour and all mice stably maintained weight gain. Rodent trials frequently supplement fibre at 5–20% weight/weight of feed and the translation of these doses in humans is often unattainable. For example, 5% of the diet as fibre is at least 20 grams/day for adult humans. It is noted that the Scientific Advisory Committee on Nutrition (SACN) recommends that human should be aiming for over 30 grams/day of dietary fibre intake[49]. If the fibre of interest is highly fermentable, e.g., inulin-type fibres, this dosage is near the top of the tolerable limit for human consumption. Consumption at this level is likely to result in unpleasant side effects such as gas, bloating, and diarrhoea[50]. Recently, Gunn et al. showed that 20 grams of psyllium can reduce gas production caused by 20 grams of inulin in a human MRI study whilst maintaining fermentation, found by in vitro testing[51]. Other fibres, such as polydextrose and soluble corn fibre, have been shown to be tolerable up to 50 grams/day in clinical trials[52]. This suggests that adding psyllium together with inulin and RS should be well tolerated in human patients, although further studies are needed.

Future studies are needed to define the mechanisms linked to the associations found in the present study, to provide evidence of the causal effects of the gut microbiota, the metabolome and immunity, and to explore the other potential dietary fibre combinations to maximise the therapeutic ratio of radiotherapy, using appropriate mouse models.

In the pelvic cancer patients, we found that they had a similar microbiota profile at phylum level to tumour-bearing mice fed the low fibre diet (0.2% cellulose). Based on our findings in mice, this implies that we could target the human gut microbiota with dietary fibre supplementation. The broad range of relative abundances of Lachnospiraceae family and Bacteroides genus among individuals, and their association with faecal metabolite production, suggests that we may be able to determine responders and non-responders to fibre supplementation in future clinical trials.

In conclusion, we demonstrated that dietary fibre supplements increased the radiation response of bladder tumours, with associated gut microbiota modification and enhancement of immune responses and metabolite levels. Since psyllium plus either RS or inulin achieved a more favourable therapeutic ratio, with improved tumour control with decreased side effects, we propose that dietary fibre
supplements may be useful adjuncts to radiotherapy in patients with pelvic malignancy. This approach has the potential to improve patient outcomes at a low cost and minimal distress to patients.

**Methods**

**Mice**

All animal experiments complied with UK Home Office Guidelines, following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. We obtained approval from the University of Oxford Animal Welfare and Ethical Review Body (AWERB), under University of Oxford project licences (PPLs) P8484EDAE and PP8415318. We used a G-Power program[53] to choose group sizes for detecting large effect sizes. C57BL/6J mice were all purchased from Charles Rivers (UK). All mice were housed in a temperature-controlled environment with a 12-h reversed-phase light/dark cycle (lights on 07:00 h) and provided with food and water *ad libitum*. We randomised the mice by using the RAND function in Excel.

**Allograft Model, Modified Diets And Irradiation Method**

At five to six weeks of age, C57BL/6 female mice were injected subcutaneously under anaesthesia with 1.5x10⁶ UPPL1591 mouse bladder cancer cells with Hanks’ Balanced Salt Solution (HBSS; Lonza) and phenol red-free Matrigel (BD Biosciences) mixture with a ratio of 1:1 at a total volume of 200 µL in the right flank. Meanwhile, they started receiving either normal chow, low fibre diet (0.2% cellulose) or high fibre diets including 5% psyllium, 5% psyllium plus 10% RS or 5% psyllium plus 10% inulin for a maximum time of 9 weeks or until they were euthanised when the tumour reached 100 mm³ or 700 mm³. Details of the diet formulae are listed in Supplementary Table 4 (Research Diets Inc, USA). Psyllium, RS, and inulin formulations were psyllium husk powder (AEP Colloids), Hi-Maize 260 RS (Ingredion) and Orafti HP inulin (Beneo), respectively. The murine bladder cancer cell line UPPL1591 was created by Dr Ryoichi Saito and was maintained in DMEM, high glucose, GlutaMAX supplement, pyruvate (Gibco) medium supplemented with 10% fetal bovine serum (Gibco). Tumour growth was measured three times a week using callipers and calculated using the following formula: axbxcxP/6. When the tumours reached 100 mm³, flank allografts were treated prone with 6 Gy of x-rays using a Gulmay-320 cabinet irradiator (300 kV, Xstrahl Inc, UK).

**Non-tumour-bearing Mice, Modified Diets And Irradiation Method**

At six to seven weeks of age, C57BL/6 female mice started receiving either a low fibre (0.2% cellulose) or high fibre diets including 5% psyllium, 5% psyllium plus 10% RS or 5% psyllium plus 10% inulin for a maximum time of 9 weeks followed by normal chow for another 12 weeks. Details of the diet formulae are listed in Supplementary Table 4 (Research Diets Inc, USA). For acute toxicity experiment, two and a half weeks after commencing the modified diets, mice were treated supine with 10, 12 or 14 Gy of x-rays (220 kVp, 13.0 mA copper filtered beam with a measured half-value layer (HVL) of 0.84 mmCu) to the
lower abdomen, including the lower small intestine using a SARRP irradiator (Xstrahl Ltd, Camberley, UK). For late toxicity experiments, two weeks after commencing the modified diets, mice were treated supine using a SARRP with 5 Gy for 5 consecutive days, using a 356-degree arc treatment and 8.5-mm collimator, with the isocentre positioned at the posterior caudal bladder wall covering the lower large intestine, to avoid the small intestine. In both experiments, small and large intestines were collected using the ‘Swiss roll technique’ described in (Moolenbeek & Ruitenberg, 1981).

**Microbiome Sample Collection And DNA Extraction From Mice**

Mouse faeces were snap-frozen in dry ice once they were collected from mouse intestines under aseptic conditions. All samples were kept at -80°C before DNA extraction. Bacterial genomic DNA was extracted using a DNeasy PowerSoil Pro DNA Isolation Kit (QIAGEN Ltd, Manchester, UK), as described previously[24] and the Human Microbiota Project[54]. All DNA samples were kept at -80°C before being sent for library preparation and sequencing at the Oxford Genomics Centre (Wellcome Centre for Human Genetics, University of Oxford, UK).

**Bacterial 16S rRNA Gene Sequencing In Mice**

16S rRNA gene sequencing methods were adapted from the methods developed for the NIH-Human Microbiota Project[54]. The amplification and sequencing of 16S rRNA gene V3V4 regions were done on a MiSeq platform (Illumina, Inc, San Diego, CA) using their 2x300 bp paired-end protocol, yielding paired-end reads with near-complete overlap. The primers containing adapters for Miseq sequencing were used for amplification and single-end barcodes, allowing pooling and direct sequencing of PCR products[55].

Raw sequence data was analysed using the QIIME2 platform, LEfSe and R packages as described previously[24]. All 16S rRNA gene-based metagenomic analyses were conducted using a QIIME2 platform[56]. After paired-end reads were merged to form consensus sequences, sequences were trimmed to a length of 300. In the taxonomic analysis, we classified the microbiota at the phylum, class, order, family, genus, and species levels by referring to the SILVA database[57]. In some cases, ‘L’ means that the classifier was unable to assign taxonomy at this level.

**Discovery Metabolomics Analysis**

Caecal or faecal contents were added to four-fold Millipore Synergy purified water at a ratio of 1:4 (caecal content:water) for homogenisation and were sent to the Department of Chemistry (University of Oxford, UK) on dry ice for discovery metabolomics analysis using ion chromatography-mass spectrometry (IC-MS). Supernatant was filtered using an Amicon ultra-0.5 centrifugal filter Unit (Merck, Cat. No. UFC500396) at 14,000 g for 25 min at 4°C and collected in total recovery vials (Waters Corporation). For the ‘allograft model, modified diets and irradiation’ and the bacterial supernatant experiments, samples were analysed as described previously[58], but the scan range was changed to 50–750 m/z. Analytes
were separated with an aqueous hydroxide ion gradient at a flow rate of 0.25 mL/min with the following steps: 0 min, 0 mM; 1 min, 0 mM; 15 min, 60 mM; 25 min, 100 mM; 30 min, 100 mM; 30.1 min, 0 mM; 37 min, 0 mM. For the 'non-tumour-bearing mice, modified diets and irradiation' experiments, analytes were separated with an aqueous hydroxide ion gradient at a flow rate of 0.25 mL/min with the following steps: 0 min, 0 mM; 1 min, 0 mM; 17 min, 40 mM; 20 min, 100 mM; 22.1 min, 0 mM; 25 min 0 mM. The presence of butyric acid was confirmed by retention time and accurate mass comparison with an authentic standard (Merck, Cat. No. CRM46975). Data processing was performed using Progenesis QI for small molecules (Waters Corp, Elstree, UK) and Metaboanalyst5.0[59]. In brief, the peak intensities table was uploaded in .csv format. Data normalisation was performed by median in addition to log10 transformation and auto scaling of the data prior to multivariate statistical analysis being performed. In the tables, metabolites tagged with ‘Accepted ID’ are based on comparison with an in-house database and therefore higher confidence because includes fragmentation pattern as a parameter. Metabolites tagged with ‘Putative ID’ are identified based on the accurate mass in Human Metabolome Database (HMDB).

**Immunohistochemistry**

Sections were deparaffinised and hydrated followed by antigen retrieval in pH 9.0 Tris/EDTA buffer using a microwave. The sections were incubated with 3% H2O2, avidin/biotin blocking kit (SP-2001), and 2.5% normal horse serum blocking solution (MP-7401; Vector laboratories). Subsequently, sections were incubated with CD8 (CST 98941; 1:400 dilution) primary antibody overnight at 4°C. ImmPRESS (Peroxidase) Polymer Anti-Rabbit IgG Reagent (MP-7401; Vector laboratories) was used as secondary antibody. The sections were visualised by DAB staining and counterstained with haematoxylin. The slides were mounted using DPX mounting medium after dehydration. Slides were digitally scanned using the Aperio ScanScope (Leica Biosystems). Cell number quantification of three parts of the tumour core for each tissue was performed on QuPath. The density of CD8+ cells within each region of interest could thus be calculated by dividing the positive-stained cell numbers by the analysed area.

**Nanostring**

Total RNA was extracted from three 5 μm sections of formalin-fixed paraffin-embedded (FFPE) tissue samples using a RNeasy FFPE kit (Qiagen, 160012457). After RNA extraction, RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, San Jose, CA, USA). Nucleic acid fragmentation was measured by an Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer System. After the hybridisation of targets, capture probes and reporter probes was completed, the cartridge was analysed at the Nuffield Department of Surgical Sciences, Oxford. Data were analysed using the nCounter mouse PanCancer Immune Profiling Panel, and data acquired with the nCounter SPRINT profiler (NanoString). Data were imported into nSolverTM analysis software v2.5 for quality control and normalisation of gene transcripts using NanoString standard analysis workflow with housekeeping genes.
**Flow Cytometry**

Mouse spleens were kept in PBS on ice and processed to obtain single cell suspensions within two hours of harvest for further flow cytometry analysis, as described previously[60]. On the day of flow cytometry analysis, the cells were stained with two panels of antibody mixtures. The myeloid panel included CD45, CD11b, CD11c, Ly6G, F4/8b and Gr-1. The lymphoid panel included CD45, CD3, CD8, CD49b, CD19 and CD4. Cell surface markers of immune cells are listed in Supplementary Table 5, and details of antibodies used are listed in Supplementary Table 6. The samples were run on a LSR II Flow Cytometer (Becton Dickinson) at the Jenner Institute, University of Oxford and analysed on Flow-Jo (Becton Dickinson).

**Plasma CD4+ T-helper cell cytokine assay**

Blood was withdrawn from mice under terminal anaesthesia by cardiac puncture using a needle rinsed with heparin and transported on ice for centrifugation. Plasma samples were then stored at -80°C before downstream analysis. Cytokine and chemokine concentrations in the supernatant (pg/mL) were measured using MILLIPLEX mouse 16-plex (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-21, IL-22, IL-23, IL-27, GM-CSF, IFNγ, TNFα), IL-18 single plex and TGFβ1 single plex magnetic bead kits (Millipore, MA). The plate was prepared as per the protocol described in[61] and was read on a Luminex 200 instrument (Thermo Fisher) at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Oxford.

**Crypt Assay**

Swiss rolls were made from small (three consecutive sections) and large (one section) intestines, and a modified crypt assay as described previously [33] was applied to quantify the acute crypt damage after ionising radiation. Briefly, regenerating crypts (presence of > 10 cells arranged in a distinct shape with no sign of apoptosis) were blind counted. The control number of crypts per length of small intestine was determined from the mean of three mock-treated mice. The percentage of surviving crypts in each group was calculated by dividing number of regenerating crypts per mm by number of control crypts per mm.

**Histopathological Examination**

Five µm-thick, formalin-fixed, paraffin-embedded (FFPE) sections from large intestine, arranged as ‘Swiss rolls’ were stained with haematoxylin & eosin (HE) and examined by two board-certified Veterinary Pathologists (ASB and SLP). Sections were histopathologically assessed and graded for the presence of inflammatory changes using a previously described scoring system[62] and the INHAND guide for non-proliferative and proliferative lesions of the gastrointestinal tract of the mouse[63]. Histopathological assessment was performed blind to experimental grouping using a conventional light microscope (Olympus BX43). Tissue sections were examined individually by ASB & SLP and in case of discordance in diagnosis a consensus was reached using a double-head microscope. The presence of fibrosis was
assessed by identifying expansion of any of the intestinal layers by bands of variably cellular, collagenous-rich connective tissue using H&E and Masson's Trichome stained tissue sections.

Cell Lines And Irradiation Method

The RT112 human bladder carcinoma cell line was obtained from DSMZ (Germany) and cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen). The T24 human bladder cancer cell line (ATCC, USA) was cultured in McCoy's 5A medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen). All cell lines were cultured in a humidified atmosphere of 5% CO₂ at 37°C and sub-cultured by washing the cells with phosphate buffered saline pH 7.4 (PBS; Gibco) followed by incubation with 0.25% Trypsin-EDTA solution (Gibco) to make new passages at around 90% confluency. Once passaged 10 times, a new batch of cells was thawed and cultured. A stock of cells was kept in -80°C freezer in fetal bovine serum supplemented with 10% dimethyl sulfoxide. All cells used in experiments tested mycoplasma negative. For ionising radiation, cells were irradiated in complete medium at a dose rate of 1.5 Gy/min using a Gamma-Service Medical GmbH GSR D1 irradiator.

Bacterial Strains And Their Supernatants

The bacterial supernatants were prepared as described previously[24]. All bacterial strains were obtained from DSMZ-German collection of microorganisms. Three strains of bacteria, namely B. acidifaciens (BA; DSM 15896), Bifidobacterium animalis (Bif; DSM10140), and F. prausnitzii (FP; DSM17677), and two cross-feeding combinations (BA+ FP and Bif+ FP) were cultured in Gifu Anaerobic Broth, Modified (GAM; Nissui Pharmaceutical, Japan). The supernatants were neutralised to the same pH as GAM broth (pH 7.2) by adding a minimal volume of 3M NaOH or 3M HCl.

Colony Formation Assay

RT112 cells were seeded at appropriate densities in triplicate, treated with bacterial supernatants for 24 hours, and irradiated with 0, 2, 4, 6, or 8 Gy. After culturing for 10 days, colonies were stained and quantified as described previously[24].

Cell Survival Analysis

The MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to assess RT112 cell viability. After the cells were seeded and cultured overnight, they were treated with bacterial supernatants for one to three days. At the end of the experiment, they were incubated in 0.45 mg/mL MTT (Life Technologies) at 37°C for 30 minutes. The absorbance at 595 nm of MTT-formazan was detected spectrophotometrically using an POLARstar Omega Microplate Readers (BMG Labtech). The
percentage of cell viability was determined by normalising the absorbance value in each condition to the mock control.

**Western Blotting**

Western blot samples were prepared as described previously[64]. Protein was visualised using the following antibodies: H3K23Ac (Cell Signaling Technology, #14932), phospho-histone H2A.X (Cell Signaling Technologies, #2577), b-actin (MERCK, #A1978), anti-mouse secondary antibody (LICOR, #925-32210) and anti-rabbit secondary antibody (LICOR, # 925-68021), and imaged using a LI-COR imaging system (Odyssey).

**Immunofluorescence Microscopy And Irradiation**

RT112 cells were cultured on 10 mm No. 1 cover glasses (VWR) that had been sterilised with 70% ethanol and rinsed with PBS prior to ionising radiation. After incubation with bacterial supernatants and irradiation, cells were allowed to recover for indicated times prior to permeabilisation with 0.3% Triton X-100. Cells were fixed with ice cold 4% paraformaldehyde and blocked by incubation in 5% BSA, as described previously[64]. After incubation with primary γH2AX (mouse anti-phospho-Histone H2A.X (Ser139) IgG; clone JBW301, Millipore) and secondary (goat anti-mouse IgG, Alexa Fluor 488, ThermoFisher) antibodies, coverslips were mounted on microscopy slides using mounting reagent, Fluormount G, with DAPI (Invitrogen). Fluorescent foci were imaged using a Zeiss 710 confocal microscopy using either a 40X or 63X objective. All microscopy images were analysed with FIJI (ImageJ) software.

**Sample Collection, DNA Extraction And 16S rRNA Gene Sequencing From Cancer Patients**

Stool samples were collected from 76 pelvic cancer patients (aged 46–88 years) visiting the surgical or oncology departments of Aberdeen Royal Infirmary for treatment, after obtaining informed consent. The samples were self-collected by the patients using faecal collection paper and universal faecal collection containers, shortly before an inpatient/outpatient appointment and brought into the hospital or, in the case of 5 samples, obtained at surgery from the right colon. The samples were stored at 4°C and prepared with no additives as described previously[65]. Briefly, the faecal sample (5 g) was weighed and mixed with 10 mL of PBS solution (supplemented with 30% glycerol). An aliquot of 450 µL was stored at -70°C and used for DNA extraction while a 3 mL aliquot was stored at -25°C for SCFA analysis. Three of the samples were also processed similarly at 24-h intervals for 3 days to examine the effect of storage on the stability and composition of faecal microbiota and SCFAs.

DNA was extracted from the human faecal samples using the FastDNA Spin kit for soil (MP Biomedicals, UK) according to the manufacturer's instructions as described previously[65]. DNA concentration was
quantified by using Nanodrop (Nanodrop One C, Thermo Fisher Scientific, USA). All DNA extracted were stored at -70°C and 16S rRNA sequencing was carried out at the Centre for Genome-Enabled Biology and Medicine (University of Aberdeen, UK).

Bacterial community profiling of the human faecal samples was performed by sequencing of the 16S rRNA gene V1-V2 region on a MiSeq platform (Illumina, Inc., San Diego, CA) with v3 chemistry and 300bp paired-end reads. Region-specific primers\(^{[66]}\) including partial Illumina adapters were used for amplification of the V1-V2 region, followed by short cycle PCR addition of full-length Illumina adapters and dual barcodes. Resulting libraries were equimolar pooled and sequenced on the MiSeq platform \(^{[55]}\). The raw sequence data were analysed using the DADA2\(^{[67]}\) R package (v 3.6.0) for ASV generation, with subsequent analyses using phyloseq R package\(^{[68]}\), LEfSe and R packages as described previously\(^{[24]}\).

**Short Chain Fatty Acid Analysis From Human Samples**

SCFAs from the prepared samples were measured by gas chromatography as described previously\(^{[69]}\). Derivatisation of the samples was carried out with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and the samples were analysed using a Hewlett Packard (Palo Alto, CA, USA) gas chromatograph. Helium was used as the carrier gas and the gas chromatograph was fitted with a fused silica capillary column. SCFA concentrations were calculated relative to the internal standard two-ethylbutyrate and external standard (a standard mixture of six SCFAs in distilled water).

**Statistical analysis**

All statistical analyses were performed on GraphPad Prism version 9.0 (La Jolla, CA) or in R using the VEGAN package\(^{[70]}\). All results in *in vivo* studies are mean ± standard error and P < 0.05 is considered statistically significant. A one-way ANOVA with Dunnett's multiple comparison test was used to compare more than two groups. In the gut microbiota analysis of mouse studies, alpha diversity was compared using the Mann-Whitney test. The LEfSe method of analysis was applied to determine the difference in faecal taxa, using the Kruskal-Wallis test. In the gut microbiota analysis of human studies, PERMANOVA tests, as implemented by Vegan, were used to test for significant differences in Bray-Curtis distances between sample groups. Date of processing was found to have a significant effect on Bray-Curtis distance so this was added to the model for all other tests (supplementary Table 7). No other technical factor (date of DNA extraction, processing time, days before DNA extraction, extraction kit) was found to have any effect on sample composition. Significantly different taxa presented from the previous comparison were used as input for LDA, which produced an LDA score. All analyses were conducted in QIIME2 or R. All data in *in vitro* studies are representative of 3 independent biological replicates unless otherwise stated, with results shown as mean and standard deviations. One-way ANOVA with Dunnett's multiple comparison test was performed to analyse the data of western blots and MTT assays. Two-way ANOVA with Dunnett's multiple comparison test was used to analyse the linear quadratic survival curves in the colony formation assay.
Declarations

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Authors’ contribution

CKT performed the animal experiments, collected and processed mouse samples, performed the analysis and interpretation of the data, and drafted the manuscript. AM assisted in the drafting of the manuscript. DS performed the immune cell quantification and analysis. ASB and SLP performed the histopathological examination. XW and KRF produced the bacterial supernatants. RS developed the UPPL1591 cell line. MDM and JSOM performed the metabolomics analysis. RAM and SH consented patients and KSN, NH, SLM and JMW co-ordinated patient sample processing. AM, DB and SMH processed the faecal samples. MG, JSR, LMS and GR approached patients for entry into the study and co-wrote the grant application with AEK. EES, ZH and ESRCD sequenced the faecal bacteria and analysed the data. SP, KAV and AEK supervised the work. AEK (and others) revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated from mouse samples supporting the conclusions of this article are available in the Figshare repository, https://gshare.com/projects/Dietary_fibre_radiotherapy_and_bladder_tumour/153165. Raw data from the human 16S sequencing are deposited in the SRA, accession: PRJNA935280.

Ethics approval and consent to participate

All animal protocols were approved by the University of Oxford Clinical Medicine Animal Welfare Ethics Review Board and conducted under animal project licences (PPL) P8484EDAE and PP8415318. Human
samples were collected under NHS Grampian Biorepository ethical approval, IRAS Ref 296502.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

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Psyllium plus RS and psyllium plus inulin cause increased tumour growth delay in bladder cancer cell allografts. (a) Treatment of UPPL1591 allografts with normal chow, low fibre (0.2% cellulose) and high fibre diets including psyllium, psyllium plus RS or inulin (n=6 for each group). Slopes of tumour curves
were calculated by linear regression to represent tumour growth rates. (b) Kaplan–Meier survival curve of mice with UPPL1591 allografts showing plots of time to tumour volume of 100 mm$^3$. (c) Phylogenetic composition of the faecal microbiota when tumours reached 100 mm$^3$. (d) Relative abundance of *Bifidobacterium animalis* of high fibre groups compared to psyllium plus inulin group. (e) Principal coordinate analysis of faecal microbiotas using Bray-Curtis dissimilarity. (f) IHC staining of CD8$^+$ cells to assess the numbers of cytotoxic T cells in tumours (n=6/group) and the representative images. (g) NanoString analysis of CD8$^+$ cells over T cells to assess the populations of cytotoxic T cells in tumours (n=3/group). One-way ANOVA with Dunett's multiple comparison test was used to compare the means of different dietary groups. Data are presented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.
Psyllium plus RS or psyllium plus inulin enhanced tumour control combined with IR in bladder cancer cell allografts. (a) UPPL1591 bladder cancer cells were injected subcutaneously in the flank of C57BL/6 mice on the same day that they started on a low or high fibre diet \((n=6/\text{group})\). Tumours were irradiated with 6 Gy IR when they reached 80-100 mm\(^3\) and monitored until 700 mm\(^3\). (b) Body weight changes of mice fed with modified diets across the whole experiment. The weight on the first day of tumour inoculation
was set to 100%. (c) Growth curves of tumours that were irradiated with 6 Gy when they reached 80-100 mm$^3$ and monitored until 700 mm$^3$. Day 0 was the day of tumour inoculation and when the mice started taking modified diets. The overall growth curves of non-irradiated and irradiated mice were plotted in solid and dotted lines, respectively. (d) Individual tumour growth curves stratified into non-IR and IR. Solid lines were mean of tumour growth curves of non-IR mice and dotted lines were individual growth curves of IR mice. Slopes of tumour curves were calculated by linear regression to represent tumour growth rates. (e) Phylogenetic composition of faecal microbiota when tumours reached 700 mm$^3$ (n=5/non-IR cohort, n=10/IR cohort in each dietary group). (f) Correlation between the *Lachnospiraceae* family relative abundance versus the tumour growth in non-IR and IR cohorts of psyllium plus inulin. Tumour curve slopes were calculated by linear regression to represent tumour growth rates. (g) Correlation between the *Bacteroides* species relative abundance versus the tumour growth in IR cohort of psyllium plus RS. One-way ANOVA with Dunett's multiple comparison test was used to compare the means of different dietary groups. Data are presented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.
Figure 3

Modulation of local immune responses and caecal metabolites profile by psyllium plus either inulin or RS. (a) Immune cell profiling and (b) pathways of NanoString platform were used to study the local tumour immunity (n=6/group). (c) Immune cell profiling in responders and non-responders to IR in psyllium plus inulin groups (n=3/group). (d) Correlation of splenic cytotoxic T cells versus tumour growth rates in the IR cohort of psyllium plus inulin group. Correlations between the Clostridia and Lachnospirales orders versus the tumour growth rate and population of splenic cytotoxic T cells in the IR cohort of the psyllium plus inulin group. (e) Principal component analysis of caecal metabolites of different dietary groups. A notable clustering effect by diets was seen in the metabolites. (f) Caecal isoferulic acid levels normalised by median in all dietary groups. (g) Correlation between the caecal
isoferulic acid level and tumour growth rate in the IR cohort of the psyllium plus RS group. Data are presented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

Figure 4

Psyllium plus RS or psyllium plus inulin mitigated the radiation injury from 14 Gy in intestinal crypt assays. (a&b) Overview of acute normal tissue toxicity experiment. Two weeks after starting low or high
bre diets, C57BL/6 mice were treated supine with 10-14 Gy SARRP IR to their lower abdomen (n=21/group). Tissues were collected 3.75 days after IR to assess the acute normal tissue responses. (c) Small intestinal crypt assay survival for modified diets and IR (n=6 per group, except for 0 Gy IR: n=3). Data were normalised to mean crypts per mm of three mock samples. (d) Caecal SCFAs in non-tumour-bearing mice after 3-week modified diet. (e) Overview of late normal tissue toxicity experiment. C57BL/6 mice were treated supine with 5 Gy SARRP IR head down for 5 consecutive days to their lower abdomen after 2-weeks of modified diet. (f) Treatment plans of CT images centred on the beam with mice positioned head down while mice were positioned upside down to avoid exposing small intestines to IR. (g) Body weight change within 2 weeks after SARRP IR. Overall comparison of body weight change for mice fed different modified diets with or without IR. Individual body weight changes of IR mice compared to their non-IR cohort in each dietary group within 2 weeks after IR. The x-axis was the days after starting the modified diets. One-way ANOVA tests were conducted for comparison of IR cohorts to non-IR cohorts at 1- and 2-week post IR. Data are presented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.
Figure 5

Bacterial supernatants from the cocultures of *B. acidifaciens* and *F. prausnitzii* conferred stronger anti-tumour phenotypes in bladder cancer cells. (a) The five bacterial supernatants used in this experiment. (b) Western blot analysis of histone acetylation (N=3) of RT112 cells treated with different bacterial supernatants. Histone acetylation levels were determined after treating with 100 mL bacterial supernatants in 2 mL of medium for 24 hours. (c) The cell survival of RT112 cells treated with 200 mL of...
GAM broth or bacterial supernatants in 500 mL of medium for 2 days (N=3). (d) Immunofluorescence microscopy analysis of γ-H2AX levels (N=3) in RT112 cells treated with 100 mL bacterial supernatants in 2 mL of medium for 24 hours. DNA damage was evaluated after treating with 2 Gy IR. (e) Linear quadratic survival curves of RT112 cells treated with 100 or 400 mL bacterial supernatant from BA+FP for 24 hours before receiving irradiation of 0-8 Gy (N=3). (f&g) Principal component analysis of known metabolites of different bacterial supernatants. Relative levels of metabolites enriched in the bacterial supernatant of BA+FP. pHs of GAM broth and bacterial supernatants were all neutralised to 7.2. BA+FP denotes the co-culture of B. acidifaciens and F. prausnitzii, while Bif+FP denotes the co-culture of Bifidobacterium and F. prausnitzii. Data are presented as mean ± SD. *p<0.05; **p<0.01; ***p<0.001.
Figure 6

Comparison of gut microbiota from pelvic cancer patients and tumour-bearing mice treated with a low fibre diet. (a) Relative abundances of bacteria at the phyla level between cancer patients and mice fed on different diets. (b) Linear discriminant analysis (LDA) scores computed for differentially abundant taxa in the patients with either high or low faecal acetate, propionate and butyrate concentrations. Median of the three SCFAs combined was the cut off between high and low levels. The alpha value was 0.05 for the
Kruskal-Wallis test and length of bar indicates the effect size associated with a taxon. (c) Correlation between Lachnospiraceae family versus the total concentration of three major gut microbiota produced SCFAs, acetate and butyrate.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- ThenetalMicrobiomesupplementary10Mar2023.pdf