The gut microbiota may drive the radiosensitising effect of a high fibre diet

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Abstract

Background: Patients with pelvic tumours, including bladder, cervix and rectal cancers, receiving radiotherapy are often given additional radiosensitising chemotherapy to improve cure rates, at the expense of increased toxicity. With an ageing population, new approaches to radiosensitisation are urgently required. Inhibition of Histone deacetylase (HDAC) is a promising mechanism of radiosensitisation. Tumour cells may accumulate butyrate which is produced by the gut microbiota via fibre fermentation to sufficient levels to cause HDAC inhibition, due to the Warburg effect. We hypothesised that mice fed a high fibre diet would have improved tumour control following ionising radiation, compared to mice fed a low fibre diet, and that the effects of the diet would be mediated through the gut microbiota.

Results: The faecal (n=59) and caecal (n=59) microbiomes from CD1 nude mice, implanted with RT112 human bladder cancer cell line flank xenografts at the same time as starting low and high fibre (soluble, insoluble, mixed) diets, were similar and they shared the three major taxa (> 80% abundance): Bacteroidales, Clostridiales and Verrucomicrobiales. Significantly higher abundance of Bacteroides acidifaciens (p<0.01) was seen in the gut microbiome of the soluble high fibre (HF) group after 2 weeks of diet. Principal coordinate analysis showed a notable cluster effect within groups, indicating that the diets indeed modified the gut microbiome. Survival analysis by log-rank test showed soluble HF conferred survival benefits, with delayed tumour growth after irradiation (n=32, p=0.005). Comparison of the gut microbiomes in the soluble HF group between responders (n=4) and non-responders (n=4) to radiation revealed significantly higher abundance of B. acidifaciens in responding mice (p<0.05). Predictive metagenomic profiling showed the gut microbiome in responders was enriched for carbohydrate metabolism. To investigate the correlation between specific bacterial taxa and tumour response to radiation, all mice fed with different diets (n=32) were
pooled together, and univariate linear regression revealed a statistically significant positive correlation between the survival time of mice and abundance of \( B.\ acidifaciens \) (\( R^2 = 0.5278, P < 0.001 \)).

**Conclusions:** High fibre diets sensitised RT112 xenografts to radiation by modifying the gut microbiome and this phenotype was positively correlated with \( B.\ acidifaciens \) abundance. These findings might be exploitable for improving radiotherapy response in human patients.

**Keywords (up to 10):** Gut microbiome, dietary fibre, inulin, cellulose, HDAC inhibition, radiotherapy, radiosensitisation, pelvic tumour, \( B.\ acidifaciens,\ Parabacteroides \)
**Background**

Patients with pelvic tumours, including bladder, cervix and rectal cancers who are receiving radiotherapy are often given additional radiosensitising chemotherapy to improve cure rates, at the expense of increased toxicity in local organs and tissues [1, 2]. With an ageing population, new approaches to radiosensitisation are urgently required. One such approach might be to modify the intake of dietary fibre by supplements before and during radiotherapy or current standard chemoradiation schedules, which would be a very cost-effective strategy, not expected to add to normal tissue toxicity [3, 4].

A high fibre diet has been shown to significantly increase the numbers of short chain fatty acid (SCFA)-producing bacteria in the intestine and faecal levels of SCFA [5], including butyrate, through fermentation of the fibre by the gut microbiota. Butyrate is involved in cellular activities which include mitochondrial activity, G-protein coupled receptors, microbiome homeostasis and histone deacetylase (HDAC) inhibition [6]. Butyrate inhibits HDAC1 and HDAC2 and leads to histone hyperacetylation [7]. Butyrate also stimulates the activity of histone acetyltransferase (HAT) by increasing the essential cofactor acetyl coenzyme A (Acetyl-CoA) [8]. In normal colonocytes, butyrate can act as the primary energy source, which provides about 70% of ATP production, via mitochondrial β-oxidation [9]. However, in cancer cells, decreased use of the Kreb’s cycle, along with upregulation of the pentose phosphate pathway and fatty-acid synthesis, results in more butyrate accumulating in the nucleus, to a level sufficient for histone deacetylase inhibition [8].

In mice, high fibre intake has been demonstrated to significantly increase butyrate levels in plasma and tumours [10] and inhibition of HDAC has been proposed as a mechanism of
increasing the response of tumour cells to radiation (radiosensitisation). Use of HDAC inhibitors can result in acetylation of histone and non-histone proteins and can inhibit the DNA damage response [11]. We previously found that in mice, the pan-HDAC inhibitor, panobinostat, in combination with ionising radiation (IR) delayed the growth of RT112 bladder cancer xenografts but did not worsen local acute (3.75 days) and late (12 weeks) toxicity in the surrounding intestinal tract [12]. The sparing of normal tissue in the gut relative to the tumour is commensurate with the normal gut colonocytes using butyrate as their primary energy source, so butyrate cellular concentrations do not accumulate to levels which can lead to HDAC inhibition.

As a proof of principle of efficacy of this approach, we tested the hypothesis that mice fed a high fibre diet would have improved tumour control following ionising radiation, compared to mice fed a low fibre diet, and that the effects of the diet would be mediated through the gut microbiota. The aims of this study were: (a) to evaluate the tumour growth in mice treated with high or low fibre diets; (b) to examine the impact of the diet on the microbiome before and after irradiation, and (c) to correlate diet-induced microbiome changes with tumour growth and response to radiation treatment.
Results

The environmental microbiome had minimal impact on gut microbiome analysis and dietary fibre content did not affect tumour growth to 50 mm³.

Female CD1 nude mice were injected subcutaneously with RT112 bladder carcinoma cells, and at the same time, they started to receive a modified diet, namely, one of the following: low dietary fibre (0.2% cellulose), low fibre with butyrate in drinking water, high soluble fibre (10% inulin), high insoluble fibre (10% cellulose) and high mixed fibre (5% cellulose, 5% inulin) (Figure 1A and Table S1). By quantification of bacterial loads using PCR and gel electrophoresis, compared to known E. coli colony forming unit (CFU) numbers, our mouse samples contained more than 10⁴ bacterial CFUs which appeared to override contaminating species in the sample microbial communities (Figure 1B). The PBS negative control was processed identically to the luminal content and tissue samples from the start of the DNA extraction. The nucleic acid amount detected in the PBS negative controls was extremely low, compared to that in the gut microbiota (Figure 1C). Furthermore, the community microbiome in this negative control was very different from the gut microbiome of the mice (Figure 1D). Therefore, the environmental microbiome had minimal impact on the analysis of the gut microbiomes of interest in this study.

By investigating samples collected when the tumours reached 50 mm³ and 350 mm³, the faecal (hereinafter called gut microbiome) and caecal microbiome were found to have similar bacterial components (Figure S1). The mice were culled when their tumours reached 500 mm³. Butyrate levels in the faeces, validated by high-performance liquid chromatography (HPLC), were generally higher in the low fibre with butyrate and high soluble fibre groups (p=NS; Figure 1E). The mean time for tumours to reach 50 mm³ was 12.8 ± 1.4 days (Figure 1F).
The landscape, diversity and enrichment of bacterial taxa of the gut microbiome in samples collected when the tumours reached 50 mm³

Faeces were collected from culled mice when the tumours reached 50 mm³. Abundance analysis revealed the five bacterial taxa with the highest abundance were *Bacteroides acidifaciens*, *Parabacteroides*, *Akkermansia muciniphila*, *Lachnospiraceae* and S24-7 (Figure 2A). In terms of alpha diversity, the soluble high fibre group had a lower Shannon’s index (p<0.001) (Figure 2B). This could be due to the higher abundance of *B. acidifaciens*, which lowered the diversity within groups. In terms of beta diversity, principal coordinate analysis showed a notable cluster effect among different groups, which indicates that samples within groups were more similar to each other than to those from the other groups (Figure 2C). This suggested that the gut microbiome was indeed modified in this study, which might be a diet-effect or a cage-effect (see later). Regarding the abundance of specific taxa in different diet groups (Figure 2D), the high soluble fibre diet significantly increased *B. acidifaciens* abundance (p<0.001); the low fibre diet increased *Parabacteroides* abundance (p<0.001), the low fibre diet with butyrate increased *Akkermansia muciniphila* abundance (p<0.001), and the high mixed fibre diet increased *Lachnospiraceae* abundance (p=0.005).

The landscape, diversity and enrichment of bacterial taxa of the gut microbiome in samples collected when the tumours reached 350 mm³

When the tumours reached 350 mm³, abundance analysis of the gut microbiome of both IR and non-IR cohorts revealed that the top 6 bacterial taxa with the highest abundance were S24-7, *Akkermansia muciniphila*, *Bacteroides*, *Lachnospiraceae*, *Clostridiales* and *B. acidifaciens* (Figure 3A). In terms of alpha diversity, the soluble HF group had a significantly
lower Shannon’s index (p<0.001 for Kruskal-Wallis test) (Figure 3B). In terms of beta diversity, principal coordinate analysis using Bray-Curtis dissimilarity showed a notable clustering effect among different groups, which indicates that samples within groups were more similar to each other than to those from the other groups (Figure 3C). An increased *Firmicutes/Bacteroides* ratio is considered to represent radiation-induced gut dysbiosis [13, 14]. Interestingly, only the low fibre group treated with radiation had a trend towards an increased *Firmicutes/Bacteroides* ratio compared to non-irradiated control, but this phenomenon was not seen in the high fibre diet groups, implying that dietary fibre may be protective and therefore reduce gut dysbiosis caused by radiation treatment (Figure S2A).

The diets continued to affect the bacterial composition of the gut microbiome as mice got older, up to the time the tumours reached 350 mm³. The taxonomic cladogram of LEfSe (Linear discriminant analysis Effect size) of the gut microbiome showed that the high soluble fibre diet increased S24-7 (Figure S2B). Studying the highest abundance bacterial taxa found in the first cohort in this cohort, the relative abundance of *B. acidifaciens*, an acetate-producing bacteria [15, 16], was distributed evenly in different diet groups, except in the soluble HF group with treated with radiation (p=0.200 for Kruskal-Wallis test) (Figure 3D). For the highest abundance bacterial taxa in the second cohort, the relative abundance of *Bacteroidales S24-7* was significantly higher in the mixed HF and soluble HF groups compared to LF and insoluble HF (p=0.001 for Kruskal-Wallis test) (Figure 3E).

**Soluble high fibre causes increased growth delay in irradiated bladder cancer cell xenografts and responses are influenced by the gut microbiota composition.**

To investigate the effect of different diets on the tumour response in mice irradiated when the tumour had grown to 50 mm³, tumour growth was monitored to 350 mm³. Slopes of the
tumour growth curves were obtained using linear regression to indicate the tumour progression rates. The high soluble fibre diet group had the lowest growth rate. The slopes were 4.4 ± 1.3 for LF, 16.1 ± 1.7 for mixed HF, 28.7 ± 1.3 for insoluble HF and 0.4 ± 1.5 for soluble HF (Figure 4A and Figure S3 for individual irradiated mouse tumour growth curves).

Kaplan Meier survival curves for time to treble tumour volume showed that the soluble HF group had the longest median survival time (7.5 days for LF, 7 days for mixed HF, 10 days for insoluble HF, 11.5 days for soluble HF; p=0.005, log-rank test) (Figure 4B).

Among the eight mice fed the soluble high fibre diet, four mice were classified as responders as they had shallower slopes to the tumour growth curves; their slopes were 7.3 ± 1.2, -0.9 ± 0.6, -4.8 ± 0.5, -5.2 ± 0.9. The other four mice were classified as non-responders with steeper slopes to the tumour growth curves, namely 34.6 ± 3.0, 31.1 ± 2.6, 23.3 ± 1.0, 33.8 ± 2.9 using linear regression (Figure 4C). In terms of alpha diversity, no significant difference in Shannon’s index was shown between responders and non-responders (Figure 4D). In terms of beta diversity, principal coordinate analysis of Bray-Curtis dissimilarity showed the gut microbiome of responders and non-responders were more similar within groups than between groups (Figure 4E).

**Differences in composition of the gut microbiome between responders and non-responders**

Linear discriminant analysis showed that the mice responding to the soluble high fibre diet for a slower tumour growth rate were enriched with *Bacteroidaceae, Flavobacterium, Flavobacteriales, Lactococcus, Streptococcus, Streptococcaceae, Allobaculum, Erysipelotrichales*. The non-responding tumour-bearing mice were enriched with *Bifidobacterium, Bidifobacteriaceae, Bifidobacteriales, Parabacteroides,*
Porphyromonadaceae, Lactobacillus, Lactobacillaceae and Lactobacillales (Figure 5A). In terms of effect size, *B. acidifaciens* and *Bacteroidaceae* had the largest enrichment in responders, and *Parabacteroides* and *Porphyromonadaceae* had the largest enrichment in non-responders (Figure 5B). To further explore these findings, the discrete false-discovery rates within all taxonomic levels were calculated (Figure 5C). In responders, *B. acidifaciens* species and the *Allobaculum* genus and in non-responders *Lactobacillus* and *Parabacteroides* had p-values <0.05, highlighting the significance of these findings. The *B. acidifaciens* abundance was significantly higher in responders than that in non-responders (p=0.29) (Figure 5D), while the *Bacteroidales S24-7* abundance was similar between responders and non-responders in the soluble HF group (p=0.200) (Figure 5E).

**Correlation between the abundance of *B. acidifaciens* or *Parabacteroides* genus and mouse survival time in IR and non-IR cohorts**

As *B. acidifaciens* was the ‘top hit’ for responders in the soluble HF group, we explored how specific bacterial taxa affected mouse survival time. The correlation between *B. acidifaciens* abundance and time to culling was investigated across the diet groups. Some mice in the non-IR cohort lived as long as those in the IR cohort, ie. >40 days, which may be a reflection of 6 Gy being a relatively low dose of radiation (Figure S4A). In the IR cohort, the time of culling positively correlated with *B. acidifaciens* abundance ($R^2=0.528$, p<0.001). However, a similar correlation was not seen in the non-IR cohort ($R^2=0.085$, p=0.357). Using the time for tumours to treble in volume as the outcome measure, mice with high *B. acidifaciens* abundance had a significantly prolonged median survival time (Log-rank test: p<0.001) (Figure 6A). A similar finding was seen in the IR cohort (p=0.003), but not in the non-IR cohort (p=0.236). To further identify specific unfavourable bacterial taxa which might offset the effect of radiation, the
Parabacteroides genus, was selected, as one of the top two ‘hits’ for non-responders in the soluble HF group. This was investigated for the association between its abundance and time to culling (Figure S4B). In the IR cohort, the time to culling negatively correlated with the abundance of Parabacteroides genus (Figure S4B; $R^2=0.164$, $p=0.022$). However, a similar correlation was not seen in the non-IR cohort ($R^2=0.084$, $p=0.360$). Using the time for tumours to treble in volume as the outcome measure, mice in the low Parabacteroides genus abundance group had no significant difference in median survival time compared to the high abundance group (Log-rank test: $p=0.374$) (Figure 6B). B. acidifaciens ($p=0.200$ for Kruskal-Wallis test) and Parabacteroides genus ($p=0.005$ for Kruskal-Wallis test) abundance was evenly distributed among all cages, which suggested that the existence of this taxa in the gut microbiome was not a cage-specific effect (Figure S5 and Table S2).

Metagenomics functional prediction of the gut microbiome by response in the soluble HF group

Functional prediction at KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways level 2 revealed that the gut microbiome in responders was enriched for carbohydrate metabolism pathways and in non-responders for membrane transport pathways (Figure 7A). To further explore this at KEGG pathways level 3, enrichment of the amino sugar and nucleotide sugar metabolism pathway was shown in responders with enrichment of the ATP-binding cassette (ABC) transporter pathway shown in non-responders (Figure 7B).
In 2016, Wei et al. demonstrated that up to four-fold-higher levels of butyrate accumulated in the lymphoma flank xenograft tumours of mice fed a high fibre diet compared to a low fibre diet [10]. Butyrate has been proposed to increase histone deacetylase inhibition, which is a known mechanism of cellular radiosensitisation [8, 11]. Therefore, the gut microbiota in mice fed a high fibre diet might act as an endogenous radiosensitiser, by providing a sufficiently high systemic butyrate level.

In this study, faecal and caecal microbiomes were investigated in mice fed with low fibre diets, low fibre diets with butyrate, high mixed fibre diets, high insoluble fibre diets and high soluble fibre diets, and profiles of both microbiomes were correlated with each other. The gut microbiomes were shaped in mice fed with different modified diets for two weeks and homogeneous gut microbiomes were seen in samples within groups. A distinct bacterial taxon was seen in each group: enrichment of *B. acidifaciens* in soluble HF, *Parabacteroides* in LF, *Akkermansia muciniphila* in LF with butyrate, and *Lachnospiraceae* in mixed HF. To date, there is only limited literature studying the effects of dietary fibre intake or the gut microbiota on tumour growth. Hardman et al. suggested that breast cancer xenografts grew more slowly in mice fed with fish oil concentrate than those tumours of control mice [17]. In contrast, Cougnoux et al. proposed that Colibactin-producing *E. coli* enhanced tumour growth of colon cancer xenografts [18]. In our study, no significant difference for the time for tumours to reach 50 mm$^3$ was seen in mice from different groups. This demonstrated that diet and the diet-modified microbiome had no effect on tumour growth, up to that point.
The tumours were irradiated when they reached a volume of 50 mm$^3$ and monitored until they reached 350 mm$^3$. As the mice aged during the time required for tumour growth, all mice were enriched for the S24-7 family. This indicated that their gut microbiomes had altered over the tumour growth period. Although the gut microbiomes became more heterogeneous, a notable cluster effect still existed in samples within groups. Mice responding to radiation in the soluble HF group were enriched with *B. acidifaciens* and non-responding mice were enriched with the *Parabacteroides* genus. A predictive metagenomics study of the gut microbiome in responders was enriched with carbohydrate metabolism pathways. This suggested that fermentation of carbohydrate, possibly soluble fibre, was enriched in responders and its final products, namely, butyrate and/or other short-chain fatty acids, might contribute to the radiosensitisation. When the mice from different diet groups were pooled, *B. acidifaciens* abundance was positively correlated with survival time and mice with high *B. acidifaciens* had the longest median survival times using Kaplan-Meier survival analysis.

Early studies have shown that dietary fibre, such as wheat bran, can be protective against colonic adenomas. Since then, systemic reviews have also provided some evidence that dietary fibre intake is inversely associated with the risk of breast cancer [19], pancreatic cancer [20], prostate cancer [21] and ovarian cancer [22]. Most of the early studies are limited to epidemiology studies. Other healthy lifestyle factors, such as exercise, abstention from smoking and alcohol, etc., rather than high fibre intake, could be major confounding biases in these studies.

More recently it has emerged that one of the major mechanisms by which dietary fibre intake protects against cancer is the modulation of the gut microbiota [23], via short chain fatty acid
production and immunomodulation. Dysbiosis has been linked to colorectal cancer, and a recent study proposed stage-distinct alterations in the tumourigenesis of colorectal cancer [24]; *Atopobium parvulum* and *Actinomyces odontolyticus* were significantly enriched in multiple polypoid adenomas and intramucosal carcinomas, and *Fusobacterium nucleatum* increased continuously from the intramucosal carcinoma to more advanced stages. *Helicobacter pylori* is the most famous and early-defined link between a bacterium and cancer [25]. Nowadays, *H. pylori* eradication has become one of the most promising treatments in the prevention of gastric cancer [26].

In this study, in CD1 nude mice, short-term high fibre intake alone and its modifying influence on the gut microbiota had no effect on tumour progression rate (to 50 mm³). Gopalakrishnan et al. demonstrated that the gut microbiota can enhance melanoma responses to anti-PD-L1, possibly via enhanced systemic and anti-tumour immunity [27]. Sivan et al. suggested that commensal *Bifidobacterium* are associated with enhanced anti-PD-L1 efficacy, via increased cytotoxic T cell accumulation in the tumour microenvironment [28]. Furthermore, the immune-stimulatory effect of CTLA-4 blockage was found to be dependent on *B. thetaiotaomicron* or *B. fragilis* [29].

All of these recent studies proposed that a modified gut microbiota can augment the efficacy of anti-tumoural treatment. However, most of them are limited to chemotherapy and immunotherapy [30]. To date, this is the first study to suggest that a high soluble fibre diet, with the related modified gut microbiome, can act as a radiosensitiser. Innate immunity plays a role in shaping the gut microbiota and adaptive immunity has a strong impact on tumour progression or treatment response. Our studies were conducted in immunodeficient CD-1
nude mice, which implies that the radiosensitising effect of dietary fibre here was independent of immunomodulation. This does not rule out the tantalising possibility that this radiosensitisation could be further enhanced by immunomodulatory effects in an immunocompetent environment. This is worthy of further investigation.

*B. acidifaciens* abundance was enhanced by a high soluble fibre diet in this study and *B. acidifaciens* was identified as a potential radiosensitiser because its abundance was positively correlated with tumour response to radiation and survival time in the IR cohort. This bacterium was first isolated in 2000 and was so named because its reduces the pH level of peptone-yeast broth with Fildes’ digest [16]. Consistent with our findings, Marques et al. demonstrated that a high-fibre diet markedly increased the prevalence of *B acidifaciens* [31]. Another previous study also showed *B. acidifaciens* to be enriched in normal human controls, compared to patients with inflammatory bowel disease [32]. Yang et al. found that *B. acidifaciens* increased insulin sensitivity and further prevented obesity [33]. However, the effect of this bacteria on tumour growth is still controversial. A study showed that increasing *B. acidifaciens* abundance was seen in hepatocellular carcinoma which was induced by a streptozotocin-high fat diet [34]. However, *B. acidifaciens* reduces isoflavones, a chemical that is associated with increased risk of breast cancer [35]. *B. acidifaciens* was also shown to contribute to the anti-tumour effect of medicinal Gynostemma saponins [36].

In terms of the immune context, IL-6 and IL-10 have been found to be augmented by *B. acidifaciens* via enhancement of MHC class II molecules expression and also co-stimulatory molecules (i.e., CD80 and CD86) on antigen presenting cells [37]. *B. acidifaciens* has also been shown to promote IgA antibody production in the large intestine [38, 39], with a protective
effect on the mucosa of large intestine, which could be exploited to reduce normal tissue toxicity of irradiation.

We also found Bacteroidales S24-7 (or Candidatus Homeothermaceae [40] or Muribaculaceae [41]), an uncultured bacterium with limited information, to be highly abundant in our study, especially in the mixed HF and soluble HF group. The prevalence of this bacterium in humans is 20% [42]. An increased prevalence of Bacteroidales S24-7 of up to 70% was shown in the Hadza tribe of Tanzania who consume tubers containing large amounts of soluble fibre [42].

Colonic inulin fermentation in healthy humans produces much higher levels of acetate than that of propionate and butyrate [43]. Also, Bacteroidetes, including B. acidifaciens, have been proposed to produce the end products acetate, succinate and possibly propionate, but not butyrate [15, 16]. Acetate levels in plasma are 3 fold higher than that of propionate and butyrate [44], and acetate production is enhanced in tumours due to hyperactive metabolism occurring during the Warburg effect [45]. It is also worth noting that synthesis of acetyl-coA from acetate is a critical substrate to enhance histone acetylation levels by histone acetyltransferase (HAT) [45], and HDAC inhibition by acetate has been shown in several previous studies [46, 47]. These findings suggest that acetate might initiate an alternative mechanism of radiosensitisation other than via butyrate.

Integrating these findings, the mouse responders in the soluble HF group had a higher abundance of acetate-producing bacteria (B. acidifaciens) which might be able to ferment inulin to provide higher acetate levels which could then act as a substrate for butyrate production, given that acetate is necessary for butyrate production particularly in the butyryl-
CoA:acetate CoA-transferase pathway [48, 49]. We speculate that the butyrate levels could be enhanced due to cross-feeding of butyrate-producing bacteria by Bacteroides acidifaciens. Interestingly, Ramirez-Farias et al. have showed that inulin increased both acetate-producing bacteria (Faecalibacterium prausnitzii) and butyrate-producing bacteria (Bifidobacterium adolescentis) in their human volunteer study [50]. Proof-of-concept has been demonstrated by cross-feeding Faecalibacterium prausnitzii and Bifidobacterium adolescentis which enhances butyrate formation [51]. In our study, most plasma butyrate levels (with one sample showing 98 µM) and all tumour butyrate levels could not be resolved above the signal to noise of the chromatogram (limit of quantification 10 µM) but this does not rule out that the effect comes from butyrate.

We have highlighted several possible mechanisms underlying the radiosensitising effects of high soluble fibre diets or B. acidifaciens, which need further investigation in future studies: (1) increased concentrations of butyrate or other short-chain fatty acids in tumours, which can act by HDAC inhibition or via other mechanisms; (2) suppression of overgrowth of unfavourable bacteria, e.g. possibly inhibiting Parabacteroides genus; and/or (3) enhancement of anti-tumoural immunity. In this study, two findings, namely, (1) the positive correlation between the time of culling and B. acidifaciens abundance and (2) the dose-dependent effect of B. acidifaciens abundance on time for tumours to treble in volume supported the hypothesis that the action of a high fibre diet on the gut microbiota may induce radiosensitisation in mouse xenograft tumours.

Environmental contamination is an inevitable issue in microbiome studies [52]. To minimise the influence of contamination in this study, bacterial loads of samples were quantified and
appropriate negative controls were included. Bacterial loads from luminal content and tissue samples contained more than $10^4$ CFUs which overrode the environmental bacteria communities. Furthermore, the bacterial compositions of the PBS negative controls were very different from those of the study groups, so the environmental microbiome was considered not to be a major source of bias in this study.

Although a strong correlation between *B. acidifaciens* abundance and tumour response to irradiation was seen, further studies are needed to validate a causal relationship between *B. acidifaciens* and radiosensitisation of bladder and other pelvic tumours.

**Conclusions**

A high soluble fibre diet increased responses of RT112 mouse xenografts to irradiation and this phenotype was associated with higher relative abundance of *B. acidifaciens*. Human patients may respond to dietary fibre supplementation before and during radiotherapy to achieve tumour radiosensitisation via modification of the gut microbiome.
Methods

Overview of study design

In this study, we aimed to evaluate the tumour growth after irradiation in mice fed high or low fibre diets and to correlate diet-induced microbiome changes with tumour growth. Two cohorts of CD1 nude female mice were studied. Two types of samples were collected, namely faeces and caecal contents. The samples from the first cohort were collected when the tumours had grown to 50 mm³, and consisted of 15 mice in five groups (n=3 per group) fed diets comprising: low fibre (< 0.2% cellulose), low fibre with butyrate (98% sodium butyrate, Sigma Aldrich), high mixed fibre (5% cellulose, 5% inulin), high insoluble fibre (10% cellulose), high soluble fibre (10% inulin) (see Table S1 for details). These diets were formulated by Jia-Yu Ke at Research Diets, Inc. on 11/6/2018. The samples from the second cohort were collected when the tumours had reached 350 mm³ either without (n=3) or following (n=8) radiation, and consisted of four groups fed diets comprising: low fibre, high soluble fibre, high insoluble fibre and high mixed fibre. 16S rRNA gene sequencing was performed on a MiSeq platform to investigate the bacterial components of the mouse gut microbiome. Metagenomic analysis was conducted using a QIIME2 platform to identify the features (differentially abundant taxa or diversity index) that correlated to the phenotype.

Mice and mouse diets

CD1-nude female mice (Charles River Laboratories, USA 6-7 weeks) were housed in a temperature-controlled environment with a 12-h reversed-phase light/dark cycle (lights on 21:00 h) and provided with food and water ad libitum. At 7 to 8 weeks of age, mice were injected subcutaneously with RT112 bladder cancer cells (DSMZ, Germany) and started receiving either a low fibre diet (2 g cellulose/3850 kcal), a high insoluble fibre diet (100 g cellulose/3850 kcal), a high soluble fibre diet (100 g inulin/3850 kcal) or a high mixed fibre
diet (50 g cellulose + 50 g inulin / 3850 kcal) for a maximum time of 9 weeks or until they were culled when the tumours reached 350 mm$^3$. Faeces, caecal contents, and proximal and distal colons from the first cohort were taken when the tumour reached 50 mm$^3$ (each group n=3) without irradiation to investigate the microbiome at baseline. Faeces and caecal contents from the second cohort were taken when the tumour reached 350 mm$^3$ after IR (each group n=8) or without IR (each group n=3) or at the end of study (9 weeks after xenograft) to study the association between the gut microbiome composition and tumour response.

**Xenograft model and irradiation method**

Mice were injected subcutaneously under anaesthesia into the right flank with 5 x 10$^6$ human bladder cancer cells (RT112) in RPMI medium (Sigma Aldrich) with phenol red-free Matrigel (BD Biosciences) at a total volume of 100 µl (1:1 ratio cell suspension to Matrigel). Tumour growth was measured three times a week and size determined by calipers using (length x width x height x π/6). To assess the effects of different dietary fibres on tumour growth after irradiation *in vivo*, mice received ionising radiation to the tumour (6 Gy, single fraction, 300 kV, using a Gulmay-320 cabinet irradiator, Xstrahl Inc, UK). A dose of 6 Gy was chosen due to the promising effect in our previous radiosensitisation experiments with a HDAC inhibitor (manuscript in preparation.)

**Microbiome sample collection and DNA extraction**

All samples were transported on ice and kept at -20°C for less than 2 hours before DNA extraction. Bacterial genomic DNA was extracted using a DNeasy PowerSoil DNA Isolation Kit (QIAGEN Ltd, Manchester, UK), as per the Human Microbiome Project [53]. Briefly, by adding sodium dodecyl sulfate (SDS), microbial cells were lysed by mechanical disruption with a ceramic bead set on 3,000 rpm for 10 minutes, followed by binding of DNA tightly to a silica membrane in a Spin Filter device at high salt concentrations. Eventually, DNA was collected
into sterile elution buffer and quantified using a NanoDrop spectrophotometer. All DNA
samples were kept at -80°C. All samples were collected and handled in sterile containers and
equipment to minimise contamination. Those sent for sequencing (Omega
Bioservices, Georgia, USA) were dried in an Eppendorf concentrator 5301 (Eppendorf North
America Inc, USA) at a rotational speed of 1,400 rpm and centrifugal force of 240 x g for 1
hour at 30°C.

Faecal butyrate levels quantification

Faecal samples were first homogenised in ice cold Millipore Synergy purified water.
Thereafter, 20 µL of sample or standard was taken and 10 µL of internal standard (valeric acid,
Alfa Aesar, UK) added prior to the addition of 5 µL 15% perchloric acid. Samples were mixed
and centrifuged at 12,000 g for 15 min at 4°C followed by direct injection (10 µL) of the
supernatant. High-performance liquid chromatography (HPLC) separation was carried out
using a Waters Acquity H-Class Quaternary Solvent Manager with mobile phases of 0.1%
formic acid in water (A) and methanol (B) and a gradient of 35-75% B on a Waters Acquity
CSH C18, 1.7 µm, 100 x 2.1 column. Butyrate and internal standard (IS) were detected by mass
spectrometry with a Waters Acquity TQ detector in positive electrospray ionisation mode.
Butyrate was detected with a cone voltage of 20 V at selected ion recording (SIR) of m/z 88.41
(M+H) and IS with a cone voltage of 15 V and SIR of m/z 103.2 (M+H).

Identification and quantification of bacterial DNA

The microbiota of the contents of the intestinal tracts and the intestinal wall of the proximal
and distal colon (tissue) was quantified by PCR of 16S rRNA. This was performed on genomic
DNA extracted as described above. The PCR was performed using primers - V3F
(CCAGACTCTACGGAGGAGC) and V3R (CGTATTACCGCGGCTGCTG) [54]. All primers were
purchased from Sigma. For each sample, Phire Tissue Direct PCR Master Mix (Thermo Fisher
Scientific) was used to amplify the 16S rRNA gene hypervariable V3 region (product size = 200 bp). PCR amplifications were performed using the following conditions: 98°C for 5 minutes followed by 35 cycles at 98°C for 5 seconds each, 66.3°C for 5 seconds, and 72°C for 30 seconds and a final extension step at 72°C for 1 minute. The amplification products were visualised on a 1% agarose gel after electrophoretic migration of 5 μl of amplified material. A standard curve was created from serial dilutions of *Escherichia Coli* from $1 \times 10^2$, $1 \times 10^4$, $1 \times 10^6$ colony-forming units (CFU) which was quantified by CFU assay. All samples were run in duplicate. In CFU assay, 20 μL of serial dilution of *E. Coli* was incubated onto Luria-Bertani (LB) agar plates, and colonies were counted and bacterial concentrations of the original samples were estimated after 24 hours incubation.

**Bacterial 16S rRNA gene sequencing**

16S rRNA gene sequencing methods were adapted from the methods developed for the NIH-Human Microbiome Project [53, 55]. The amplification and sequencing of 16S rRNA gene V3V4 region were done commercially by Omega Bioservices (Georgia, USA) on a MiSeq platform (Illumina, Inc, San Diego, CA) using the 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 [56]. PBS negative controls were included to eliminate the confounding effects of environmental contamination.

All 16S rRNA gene-based metagenomic analysis was conducted using a QIIME2 platform [57]. Quality filtered sequences with >97% identity were clustered into bins known as Operational Taxonomic Units (OTUs), using open-reference OTU picking. The relative abundance of each OTU was obtained from all samples. In the taxonomic analysis, the microbiome at the phylum,
class, order, family, genus and species levels was classified with reference to the Greengenes database [58].

The analysis pipeline was as follows:

i. All sequences were trimmed to a length of 240, since the quality dropped above this length based on the sequence quality plots.

ii. De-noised sequencing errors by using the “Deblur” plugin in QIIME2 [59].

iii. Taxonomic assignment was performed with Greengenes [60] by the “feature-classifier” command.

iv. To visualise the differences in microbial composition between gut contents and tissue, a taxonomic profile was generated by conducting differential abundance analysis using balances in gneiss.

v. To identify the features characterising the differences between groups, the LEfSe method of analysis was performed to compare abundances of all bacterial clades [61]. By validation using the Kruskal-Wallis test at the α setting of 0.05, effect size was obtained by LDA (linear discriminant analysis) based on the significantly different vectors resulting from the comparison of abundances between groups.

vi. To validate the significance of enrichment of bacterial taxa among different groups, discrete false-discovery rates (DS-FDR) were calculated [62].

vii. A phylogenetic tree was generated by using the “phylogeny” plugin in QIIME2.

viii. To investigate the alpha and beta diversity, the diversity commands of “alpha-group-significance” and “beta-group-significance” were used to obtain Shannon’s index, and Bray-Curtis dissimilarity. A principal coordinates (PCoA) plot was obtained by using the Emperor Tool based on the results of Bray-Curtis dissimilarities.
The OTU table was rarefied using the “alpha-rarefraction” command in QIIME2.

The alpha rarefraction plot showed the richness of the samples with increasing sequence count.

To predict the metagenome functional profiles, PICRUSt, a bioinformatics software package, was used to collapse predicted functions (KEGG Orthology; KO) based on 16S rRNA surveys into higher categories (KEGG pathway) after picking OTUs and normalisation [63].

**Statistical analysis**

Power calculations for the number of mice per group were done using G*Power software version 3.1.9.4 [64]. Alpha diversity and relative abundance of specific bacterial taxa were compared using the Kruskal-Wallis test following by Dunn’s multiple comparison test. All mice were classified into high, intermediate or low diversity groups based on tertiles of distribution.

Time to treble in volume was defined as the interval (in days) from the date of irradiation (growth to 50 mm³) to the date for the tumour to treble in volume. Tumour growth curves were analysed for each group, and their slopes were compared using one-way ANOVA. The LEfSe method of analysis was applied to determine the difference in bacterial taxa, using the Kruskal-Wallis test. Significantly different taxa presented from the previous comparison applying LEfSe method were used as input for LDA, which produced an LDA score. Volcano plots showed the significance of the taxa which are different among different groups, with log_{10}(FDR-adjusted p-values) on the y-axis and median-adjusted effect sizes on the x-axis. In addition, mice were also classified as having high, intermediate and low abundance of *B. acidifaciens* or high and low abundance of *parabacteroides* genus based on the relative abundance of these taxa in the gut microbiome sample. All analyses were conducted in QIIME2 and GraphPad Prism version 8.0 (La Jolla, CA).
## List of abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ABC | ATP-binding cassette |
| B. acidifaciens | Bacteroides acidifaciens |
| BBN | N-butyl-N-(4-hydroxybutyl)-nitrosamine |
| CFU | colony formation unit |
| E. Coli | Escherichia coli |
| FDA | false-discovery rate |
| Gy | Gray |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase |
| HF | high fibre |
| Insoluble HF | insoluble high fibre |
| IR | irradiation |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| LC-MS | liquid chromatography–mass spectrometry |
| LDA | linear discriminant analysis |
| LEFSe | linear discriminant analysis effect size |
| LF | low fibre |
| LF+B | low fibre plus butyrate |
| MIBC | muscle-invasive bladder cancer |
| Mixed HF | mixed high fibre |
| OTU | operational taxonomic unit |
| PCoA | principal coordinates analysis |
| PICRUSt | Phylogenetic Investigation of Communities by Reconstruction of Unobserved States |
| SCFA | short chain fatty acid |
| SDS | sodium dodecyl sulfate |
| Soluble HF | soluble high fibre |
Declarations

Ethics approval and consent to participate
All animal procedures were performed in accordance with the ARRIVE guidelines. All animal protocols were approved by the University of Oxford Clinical Medicine Animal Welfare Ethics Review Board and conducted under animal project licenses (PPL) P4B738A3B and P8484EDAE.

Consent for publication
Not applicable

Availability of data and material
Raw 16S rRNA reads and metadata have been made available in Figshare (https://figshare.com/projects/The_gut_microbiota_may_drive_the_radiosensitising_effect_of_a_high_fibre_diet/68393).

Competing interests
The authors declare that they have no competing interests.

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Author's contributions
CKT extracted the DNA from mouse samples, performed the analysis and interpretation of the data and drafted the manuscript. SP performed the animal experiments, collected the faeces, caecal contents, intestinal tissue and blood. AH measured the faecal butyrate levels. AEK conceived the study, supervised the work, and revised the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. The environmental microbiome had minimal impact on gut microbiome analysis and dietary fibre content did not affect tumour growth to 50 mm³

(A) Two microbiomes were analysed from the intestinal tract, namely, faecal and caecal content samples collected when tumours reached 50 mm³ and 350 mm³ respectively. (B) Quantification of bacterial load from different tissue and luminal contents from mice, with E. Coli (1 x 10², 1 x 10⁴, 1 x 10⁶ CFUs) as controls (n=1 mouse). (C) Comparison of the total amount of nucleic acid quantified by PicoGreen assay in all samples collected when the tumours reached 50 mm³ and 350 mm³. (D) Common bacterial taxa at the species level in 4 samples of PBS, as negative controls of DNA extraction by 16S rRNA sequencing. (E) Butyrate level in the faeces at the time of culling, which was between 11-16 days. (F) All mice were culled when tumours had reached 50 mm³, between 11 to 16 days after tumour inoculation, average 12.8 (STD ± 1.4) days.

Figure 2. Dietary fibre shapes the baseline gut microbiome when tumours reached 50 mm³.

(A) Stacked bar plot of phylogenetic composition of common bacterial taxa at the species level when tumours reached 50 mm³. Faeces were collected from mice fed with low fibre, low fibre with butyrate, high mixed fibre, high insoluble fibre and high soluble fibre diets (n=3 for each group). (B) Shannon’s index of gut microbiomes by Kruskal-Wallis test. Error bars represent the interquartile range of diversity scores. (C) Principal coordinate analysis of gut microbiomes using Bray-Curtis dissimilarity. A notable clustering effect by diet was seen in the gut microbiome. (D) Differentially abundant taxa when the tumours reached 50 mm³. All comparisons among different diet groups was performed by Kruskal-Wallis test and Dunn’s multiple comparison tests. All tests compared each median with the ‘control’ denoted. The diet with the highest abundance of a taxa was denoted as the control. *P<0.05; **P<0.01.
Figure 3. Composition of the gut microbiome when tumours reached 350 mm³. (A) Stacked bar plot of the phylogenetic composition of common bacterial taxa at the species level when tumours reached 350 mm³. Samples were collected from mice fed with low fibre, high mixed fibre, high insoluble fibre and high soluble fibre diets (n=8 for each group). (B) Shannon’s index of gut microbiomes by Kruskal-Wallis test. Error bars represent the interquartile range of diversity scores. (C) Principal coordinate analysis of gut microbiomes using Bray-Curtis dissimilarity. Relative abundance of (D) *B. acidifaciens* and (E) *Bacteroidales S24-7* in mice with or without irradiation.

Figure 4. Soluble high fibre causes increased growth delay in irradiated bladder cancer cell xenografts and responses are influenced by the gut microbiota composition. (A) Tumour growth in RT112 flank xenografts irradiated with 6 Gy IR, in mice fed low fibre, high mixed fibre, high insoluble fibre and high soluble fibre diets (n=8 for each group). Slopes of tumour curves were calculated by linear regression to represent tumour growth rates and compared by ANOVA. (B) Kaplan–Meier survival curves for mice with RT112 xenografts showing plots of time to treble tumour volume. (C) Mice were stratified into responders and non-responders based on the tumour response to radiation. (D) Shannon’s index of gut microbiota in responders and non-responders by Kruskal-Wallis test. Error bars represent the interquartile range of diversity scores. (E) Principal coordinate analysis of gut samples (n=8) in soluble HF group by response using Bray-Curtis dissimilarity.

Figure 5. Differences in composition of the gut microbiome between responders and non-responders. (A) Taxonomic cladogram from LEfSe showing differences of taxa in responders and non-responders of soluble HF group. Dot size is proportional to the abundance of the taxon. (B) Linear discriminant analysis (LDA) scores computed for differentially abundant taxa in the microbiomes of responders (green) and non-responders (red). Length indicates the
effect size associated with a taxon, p=0.05, Kruskal-Wallis test. (C) Discrete false-discovery rate of different abundant taxa in responders and non-responders in the soluble HF group. Differential abundance within all taxonomic levels in responders versus non-responders by Mann-Whitney U test. Dots are overlapping between Bacteroides acidifaciens and Allobaculum, and between Lactobacillus and Parabacteroides. Relative abundance of (D) B. acidifaciens and (E) Bacteroidales S24-7 and in responders and non-responders in the soluble HF group.

**Figure 6. Abundance of OTUs within the gut microbiome is predictive of response to ionising irradiation.** Kaplan-Meier (KM) plots of time for tumours to treble in volume, whole cohorts, IR cohorts, non-IR cohorts, based on (A) B. acidifaciens or (B) Parabacteroides genus abundance in IR and Non-IR cohorts from different diet groups combined. Comparison KM plots by log-rank test in mice with high abundance (green; relative abundance > 0.1), intermediate abundance (red; 0.1 > relative abundance > 0.01), or low abundance (blue; 0.01 > relative abundance) of B. acidifaciens in all, IR and non-IR cohorts. For Parabacteroides genus, relative abundance more than or equal to 0.01 was classified as high (blue), while less than 0.01 was classified as low (green).

**Figure 7. A favourable gut microbiome is associated with enhanced carbohydrate metabolism.** Metagenomic functional prediction by PICRUSt of the gut microbiome in responders (n=4) and non-responders (n=4) in the soluble HF group with reference to the KEGG database (A) level 2 and (B) level 3. Columns represent mice (responders, orange; non-responders, blue), and rows represent enrichment of predicted KEGG pathways (red, low enrichment; yellow, medium enrichment; blue, high enrichment).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7