

Title	Investigation of the gut microbiome, bile acid composition and host immunoinflammatory response in a model of azoxymethane-induced colon cancer at discrete timepoints		
Authors	Keane, Jonathan M.;Walsh, Calum J.;Cronin, P.;Baker, Kevin J.;Melgar, Silvia;Cotter, Paul D.;Joyce, Susan A.;Gahan, Cormac G. M.;Houston, Aileen M.;Hyland, Niall P.		
Publication date	2022-11-23		
Original Citation	Keane, J.M., Walsh, C.J., Cronin, P., Baker, K., Melgar, S., Cotter, P.D., Joyce, S.A., Gahan, C.G.M., Houston, A., and Hyland, N.P. (2022) 'Investigation of the gut microbiome, bile acid composition and host immunoinflammatory response in a model of azoxymethane-induced colon cancer at discrete timepoints', British Journal of Cancer, https://doi.org/10.1038/ s41416-022-02062-4.		
Type of publication	Article (peer-reviewed)		
Link to publisher's version	https://doi.org/10.1038/s41416-022-02062-4 - 10.1038/ s41416-022-02062-4		
Rights	© The Author(s),This version of the article has been accepted for publication, after peer review and is subject to Springer Nature's AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: https://doi.org/10.1038/ s41416-022-02062-4.		
Download date	2025-07-30 22:10:00		
Item downloaded from	https://hdl.handle.net/10468/13895		



University College Cork, Ireland Coláiste na hOllscoile Corcaigh

	AOM	Progression following AOM			
		Normal colon epithelium (week 8)	Normal colon epithelium (week 12)	Aberrant crypt foci (ACF) (week 24)	Adenoma (week 48)
	Microbiome	α-diversity↑ Similarity↓ Proteobacteria↑	α-diversity ↔ Similarity↓ Phyla ↔	α-diversity ↔ Similarity↓ Phyla ↔	α-diversity ↔ Similarity↓ Actinobacteria↑ Verrucomicrobia↓
	Inflammation	No change	TNF-a↑ CXCL1↑ IL-1β↑ CXCL2↑ IL-12↑ CXCL5↑	IL-6↑ TNF-α↑ IL-1β↑ IL-12↑	No change
	Bile Acids	Total↓ 1º conjugated ↔ 1º unconjugated↓ 2º conjugated↓ 2º unconjugated↓	Total↓ 1º conjugated ↔ 1º unconjugated↓ 2º conjugated ↔ 2º unconjugated↓	Total ↔ 1º conjugated↑ 1º unconjugated↑ 2º conjugated↑ 2º unconjugated↓	No change

Investigation of the gut microbiome, bile acid composition and host
 immunoinflammatory response in a model of azoxymethane-induced colon cancer
 at discrete timepoints.

- 4
- 5 Keane JM ^{1, 2, 3, 4, 5}, Walsh CJ ^{1, 6}, Cronin P ^{1, 4}, Baker K ^{3, 7}, Melgar S ¹, Cotter PD ^{1, 6}, Joyce SA^{*, 1},
- 6 ⁴, Gahan CGM ^{*, 1, 2, 8}, Houston A ^{*, 1, 3, #}, Hyland NP ^{*, 1, 5}.
- 7
- 8 1. APC Microbiome Ireland, University College Cork, Ireland
- 9 2. School of Microbiology, University College Cork, Ireland
- 10 3. Department of Medicine, University College Cork, Ireland
- 11 4. School of Biochemistry and Cell Biology, University College Cork, Ireland
- 12 5. Department of Physiology, University College Cork, Ireland
- 13 6. Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland
- 14 7. Department of Pathology, University College Cork, Ireland
- 15 8. School of Pharmacy, University College Cork, Ireland
- 16 *These authors contributed equally to this work
- 17
- 18 # Corresponding Author
- 19 Clinical Science Building,
- 20 University College Cork,
- 21 Cork University Hospital,
- 22 Wilton,
- 23 Cork,
- 24 Ireland
- 25 Email: <u>a.houston@ucc.ie</u>; Dr Aileen Houston ORCiD (0000-0003-1362-5256)
- 26
- 27 Key words: Time-course, cytokine, chemokine, bile acids, tumorigenesis, microbiota
- 28
- 29
- 30
- 31
- 32

34 Abstract

33

Background: Distinct sets of microbes contribute to colorectal cancer (CRC) initiation and 35 36 progression. Some occur due to the evolving intestinal environment but may not contribute 37 to disease. In contrast, others may play an important role at particular times during the 38 tumorigenic process. Here, we describe changes in the microbiota and host over the course 39 of azoxymethane (AOM)-induced tumorigenesis. Methods: Mice were administered AOM or 40 PBS and were euthanised 8, 12, 24 and 48 weeks later. Samples were analysed using 16S rRNA gene sequencing, UPLC-MS and qRT-PCR. **Results**: The microbiota and bile acid profile 41 42 showed distinct changes at each timepoint. The inflammatory response became apparent at 43 weeks 12 and 24. Moreover, significant correlations between individual taxa, cytokines and 44 bile acids were detected. One co-abundance group (CAG) differed significantly between PBS-45 and AOM-treated mice at week 24. Correlation analysis also revealed significant associations between CAGs, bile acids and the bile acid transporter, ASBT. Aberrant crypt foci and 46 47 adenomas were first detectable at weeks 24 and 48, respectively. **Conclusion:** The observed 48 changes precede host hyperplastic transformation and may represent early therapeutic 49 targets for the prevention or management of CRC at specific time-points in the tumorigenic 50 process.

- 51
- 52
- 53
- 54
- 55

56

58 Introduction

59 Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second 60 leading cause of cancer-related death worldwide ¹. Most cases are sporadic in nature (approximately 75%) and occur in people without a genetic predisposition or a family history 61 62 of CRC². Recent studies have implicated the intestinal microbiome in the pathogenesis of 63 CRC ³⁻⁵. In healthy subjects, the gut is primarily populated by a core microbiota composed of obligate anaerobes belonging mainly to the phyla Firmicutes and Bacteroidetes, and to a 64 lesser extent to Actinobacteria, Proteobacteria, and Verrucomicrobia ⁶. Analysis of 65 66 community diversity and richness indices based on 16S rRNA gene sequencing has shown 67 significant alterations in microbial diversity both at the site of the primary tumour and in 68 faecal samples from CRC patients ^{3, 7}. Understanding the role of the human gut microbiota in 69 colon cancer, however, has largely depended on examining patients already presenting with 70 CRC. To determine temporal changes in the gut microbiota at different stages of human 71 colon cancer development, some studies have examined the microbiota profile in patients 72 with intestinal polyps ⁸, with others examining the microbiota at different stages of the tumorigenic process ^{3, 5, 9}. Aberrant crypt foci (ACF) are thought to be the earliest identifiable 73 74 lesion in the colon carcinogenic process ¹⁰, with microbiome changes associated with ACF recently identified in a human study ¹¹. The role of the gut microbiota in the progression from 75 76 healthy to adenoma to CRC, however, is undoubtedly multifactorial and can affect the 77 various stages of the tumorigenic process. This represents a significant challenge for human-78 based studies. Further research in experimental animal models is necessary to better 79 understand the mechanisms that underlie the association between the gut microorganisms 80 and CRC.

81

82 One mechanism by which the gut microbiota may affect colon carcinogenesis is the 83 production or modification of metabolites such as bile acids ¹². Bile acids are endogenous, amphipathic molecules which facilitate uptake of dietary fats, and have been implicated in 84 85 colon carcinogenesis ¹³. For example, administration of cholic acid (CA), a primary bile acid, increased the incidence of colonic tumours in rats treated with genotoxic azoxymethane 86 (AOM)¹⁴. In contrast, ursodeoxycholic acid (UDCA) reduced tumour burden¹⁴, suggesting a 87 dual role for bile acids in the tumorigenic process. Therefore, understanding the bile acid-88 89 gut microbiome axis in the development of colon cancer may reveal a dynamic mechanism 90 by which the gut microbiota could influence cancer risk.

91

92 CRC-associated microbial communities also differentially correlate with the expression of host immunoinflammatory response genes ³. Inflammation is a well characterised risk factor 93 94 for CRC and a controlled inflammatory response is critical for immune protection against cancer¹⁵. Evidence suggests that the microbiota can influence colonic inflammation, and the 95 96 microbiota is, in-turn, influenced by host inflammatory processes, resulting in complex 97 reciprocal interactions ^{16, 17}. This is further supported by observations of microbial regulation of cytokines and chemokines in mouse models of CRC^{18, 19}. Several cytokines, including 98 99 interleukin 1 β (IL-1 β), interleukin 6 (IL-6) and tumour necrosis factor α (TNF α), have been 100 shown to protect against cancer development in some circumstances, whilst contributing to tumour initiation and progression in others ²⁰⁻²². This highlights the importance of 101 102 understanding the temporal expression profiles of these cytokines.

103

Here, we performed a time-course study in a C57BL/6J mouse strain with a prolonged period of disease onset following administration of AOM to establish the temporal sequence of events during tumorigenesis involving the microbiota, bile acid metabolism and expression of host immunoinflammatory genes. We identified distinct changes in both the immune and bile acid profiles, as well as particular microbial signatures that varied from the initial genotoxic insult to the appearance of pre-malignant disease and observed significant interactions between these factors.

112 Materials & Methods

113 Reagents

AOM (Merck, Darmstadt, Germany); RNAlater (Merck); Tetro cDNA synthesis kit (Bioline,
Nottingham, UK); SensiFAST No-ROX kit (Bioline); QIAamp Fast DNA Stool Kit (Qiagen,
Manchester, UK); nucleic acid probes from Roche Universal Probe Library; Custom oligo qPCR
primers (Eurofins Genomics, Ebersberg, Germany).

118 Animals and Study Design

Animal experiments were conducted in accordance with the regulations and guidelines of
the Irish Department of Health following approval by the University College Cork Animal
Experimentation Ethics Committee (2011/023).

122 In this study, a total of 64 female C57BL/6JOlaHsd mice (6-8 weeks of age; Envigo, 123 Blackthorne, UK) were housed in a specific pathogen-free facility on a 12-hour light/dark 124 cycle at 22°C with access to water and chow ad libitum. After acclimatisation, equal numbers 125 of mice were randomly assigned to two groups and were administered an intraperitoneal 126 (i.p.) injection of 10 mg/kg AOM to induce tumorigenesis (n = 32) each week for five 127 consecutive weeks while control mice received phosphate buffer saline (PBS; n = 32). 128 Following necropsy, each sample was allocated a random number, to which the subsequent 129 investigators were blinded. To help to prevent horizontal microbiome transmission between 130 co-housed mice, mice were group housed in two cages per treatment group (PBS or AOM) 131 per cull time. Timed culls were performed at 8, 12, 24 and 48 weeks (n = 8 per group at each 132 timepoint) following AOM or PBS administration and mice were euthanised by cervical 133 dislocation. The experimental unit was considered a single animal. Sample size was 134 calculated using g*power, with the standard deviation and magnitude of difference 135 calculated from previous studies quantifying medium to large ACF development in response 136 to AOM as an endpoint.

137 Faecal 16S rRNA Sequencing

138 DNA was extracted from faeces using the QIAamp Fast DNA Stool Kit as per the 139 manufacturer's instructions with the addition of a bead-beating step.

140 The V3-V4 variable region of the 16S rRNA gene was amplified from each extracted DNA

sample according to the 16S metagenomic sequencing library protocol (Illumina, San Diego,

142 CA, USA) and sequenced on an Illumina MiSeq. See Methods in Supplementary Material.

143 Generating Co-abundance Groups

To identify patterns in the variation of the microbiota, a set of co-abundance groups (CAGs) were determined by clustering operational taxonomic units (OTUs) by the correlation of their relative abundances. Initially, OTUs were trimmed to remove taxa present in less than 20% of samples and all non-prokaryotic taxa. A matrix of Kendall's Tau values was then generated for each pair of OTUs, and these values were clustered by Ward-linkage according to their Pearson's correlation coefficient and visualised using the *Made4* package in R. Each cluster of taxa was then assigned to a CAG ²³.

151 Ultra-Performance Liquid Chromatography – Mass Spectrometry (UPLS-MS)

Faecal samples were used for analysis of bile acids. UPLC-MS was performed as described ²⁴.
Briefly, five microliters of extracted bile acids were injected onto a 50-mm T3 Acquity column
and analysed in negative electrospray mode by an LCT Premier mass spectrometer (Waters,
Dublin, Ireland). Each analyte was identified according to its mass and retention time.
Standard curves were performed using known bile acids, and each analyte was quantified
according to the standard curve and normalised according to the deuterated internal
standards.

159 Cell Line Maintenance

HT29 cells were obtained from ATCC and maintained in DMEM supplemented with 10%
foetal calf serum (FCS), and 1% penicillin/streptomycin solution in a 37°C, 5% CO₂ humidified

- 162 incubator. Cells are routinely tested for mycoplasma contamination.
- 163 Faecal Water Preparation

Faecal water was prepared by suspending 0.3g faeces in 1mL PBS and subjected to bead beating for 15 seconds before centrifugation for 10 minutes. The supernatant was collected and stored at -20°C before use. Faeces were pooled based on treatment and timepoint to generate sufficient material. HT29 cells were serum starved (0.5% FCS) overnight and were then exposed to faecal water (1:10 dilution) for four hours.

169 Quantitative Real-Time PCR

170 RNA extraction was performed using the GenElute Mammalian Total RNA kit (Merck) as per 171 manufacturer instructions and converted to cDNA using the Tetro cDNA Synthesis Kit 172 (Bioline). Genes were amplified using primers matched to appropriate hydrolysis probe from 173 the Roche probe library (Supplemental Table 2) in a LightCycler 480 for 40 PCR cycles. 174 Relative transcription was calculated using the $2^{-\Delta\Delta CT}$ method standardised to the average of 175 the control group ΔCT^{25} . Human CXCL1 was amplified using a primer-probe combination 176 from Integrated DNA Technologies (Iowa, USA).

177 Statistics

Statistics were performed in SPSS Version 24 (Chicago, IL, USA), GraphPad version 9 (San 178 179 Diego, CA, USA) and R Version 3.5.0 using the Made4, vegan, pairwiseAdonis, 180 compareGroups, phyloseq and ggplot packages. Statistical significance was set to p<0.05. 181 Benjamini-Hochberg FDR adjustment for multiple comparisons was applied where noted, 182 with a false discovery rate set to 5%. Outliers were detected using Grubbs' test. Normality 183 was determined by the Shapiro-Wilk test. Groups were compared by two-tailed student's t-184 test or MWU-test. Where the F value was statistically significant, data were analysed using 185 the Welch t-test. For HT29 cell analysis, a one-tailed student's t-test was performed. 186 Permutational ANOVA (PERMANOVA) was used to compare β -diversity and CAGs, using unweighted Unifrac and Euclidean-squared distance matrices, respectively. Correlations 187 188 were examined using Pearson's R² and Spearman's R correlation coefficients. Throughout, asterisks denote significance where * represents p<0.05, ** p<0.01, and *** p<0.001. 189

190

192 Results

193 Macroscopic and microscopic changes in response to the carcinogen, AOM

194 Given that CRCs occur sporadically in most cases ², we chose AOM alone to mimic human 195 sporadic CRC development ²⁶. Moreover, as different mouse strains have been shown to exhibit differential sensitivity to AOM-induced tumorigenesis ²⁶, we chose C57BL/6J mice, 196 197 which display a lower sensitivity to AOM, to improve the temporal resolution of our study. 198 Consistent with this approach, no signs of hyperplastic or neoplastic transformations were 199 observed in mice at either 8- or 12-weeks post-AOM administration. Moreover, AOM-treated 200 mice gained less weight than PBS-treated mice over the course of the study, and this 201 difference in bodyweight-gain was significant at week 48 with AOM-treated mice weighing 202 on average 3.4g less than PBS-treated mice (t-test, p < 0.05). Faecal occult blood (FOB) and 203 ACF were first observed at week 24 (Table 1). FOB was also apparent in the faeces in three 204 out of eight AOM-treated mice prior to week 48. Two of these mice harboured at least one 205 colonic adenoma (Table 1).

206

207 Temporal microbiota changes in response to AOM

208 Shannon index (Figure 1a) was used to assess the α -diversity and evenness of gut microbiota 209 in faecal samples from each experimental group. The observed species (OS; Figure 1a) index 210 was used to estimate microbial richness, and the phylogenetic diversity (PD; Figure 1a) was 211 also determined at each time-point. Analysis of AOM- versus PBS-treated mice revealed that 212 the α -diversity of the microbiota was altered very early in the tumorigenic process. At week 213 8, OS and PD were significantly increased in AOM-treated mice (Figure 1a; MWU-test p<0.05 214 after FDR adjustment), suggesting that there is an increase in diversity within the AOM group 215 at this time. Beta (β)-diversity (Figure 1b), which compares samples based on overall 216 bacterial community composition across groups, also differed significantly between AOM-217 and PBS-treated mice at week 8 (Figure 1a; PERMANOVA of unweighted Unifrac, p=0.008, 218 R²=0.153). The dominant phyla in both AOM- and PBS-treated mice were Bacteroidetes and 219 Firmicutes. Although the abundance of these phyla did not change significantly, we did 220 observe a significant increase in Proteobacteria in AOM-treated mice (Figure 2a).

221

Of the significantly changed genera, the majority were among taxa corresponding to the Firmicutes, with changes also observed within Proteobacteria and Actinobacteria. At week 8, there was a significant suppression of *Lactobacillus* and an increase in *Olsenella* in AOM-

225 treated mice (Figure 3a; MWU-test, p<0.05 after FDR adjustment). Alterations in these taxa have previously been correlated with colon cancer ^{27,28}. This decrease in *Lactobacillus* could 226 227 account for some of the changes observed in the bile acid pool at this time-point due to its 228 role in bile acid metabolism, although we did not observe any correlation between bile acids 229 and these bacterial genera. Since community structure can be more informative than 230 abundance differences of individual taxa, we next analysed the microbiota by determining 231 CAGs. The taxon composition of each CAG can be found in Supplemental Table 3. However, 232 we observed no significant difference between CAGs in either PBS- or AOM-treated groups 233 at week 8 (Figure 2b; t-test and MWU-test, p>0.05 after FDR adjustment).

234

At Week 12, there were no changes in α -diversity but β -diversity differed significantly between treatments (Figure 1b; PERMANOVA of unweighted Unifrac distances, p=0.017, R²=0.149). At the phylum level, no alterations were detected (Figure 2a). There were only nine individual genera that differed significantly between PBS- and AOM-treated mice (Figure 3b). Moreover, as observed at week 8, there were no significant differences between CAGs (Figure 2b; t-test and MWU-test, p>0.05 after FDR adjustment).

241

242 By week 24, no additional changes in α -diversity were observed (Figure 1a). However, β -243 diversity differed significantly between AOM- and PBS-treated mice at weeks 24 and 48 244 (Figures 1b; PERMANOVA of unweighted Unifrac distances, p=0.001 and p=0.01, 245 respectively). Whilst there were no significant changes at the phylum level at week 24 (Figure 2a), changes were observed in specific members of Firmicutes, Bacteroidetes, 246 247 Proteobacteria and Tenericutes. Of the genera enriched in AOM-treated mice, Oscillibacter was previously associated with increased cancer risk ³ (Figure 3c). At this time-point we 248 249 observed the first significant differences in the CAGs, with CAG5 being significantly 250 decreased in AOM-treated mice (Figure 2b; t-test, p<0.001 after FDR adjustment). This CAG 251 is dominated by Bacteroidetes which comprise >90% of its abundance.

252

At week 48, there was a significant increase in Actinobacteria and a significant reduction in Verrucomicrobia in AOM-treated mice (Figure 2a). Of the significantly changed genera at this time-point, *Akkermansia* was the only member of the Verrucomicrobia phylum that was decreased (Figure 3d). Similarly, for Actinobacteria, *Bifidobacterium* was the only member of this phylum that was increased. Moreover, at this time-point we observed the greatest number of significantly altered genera (Figure 3d). These were predominantly Firmicutes
(7/21), Proteobacteria (5/21) and Tenericutes (5/21).

260

261 Bile acid metabolism following AOM administration

262 Dysregulation of bile acids has been implicated in tumorigenesis ¹³. Bile that is not reabsorbed in the small intestine is subjected to microbial transformation. First, bile salts are 263 264 deconjugated from taurine and glycine by the bacterial enzyme bile salt hydrolases (bsh) to 265 form free bile acids. Unconjugated primary bile acids (mainly cholic acid (CA), 266 chenodeoxycholic acid (CDCA) and muricholic acid (MCA) in mice) are converted into secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid (LCA), and 267 268 ursodeoxycholic acid (UDCA) by bacterial 7α -dehydroxylase. Moreover, the apical sodium-269 dependent bile acid transporter (ASBT) is expressed on the apical membrane of enterocytes 270 and mediates the reabsorption of bile acids from the intestine. With respect to the faecal 271 bile acid analysis, the concentration of total bile acids was significantly reduced in AOM-272 treated mice at week 8 (Figure 4a; t-test, p<0.01). Both unconjugated primary (CDCA) and 273 conjugated and unconjugated secondary bile acids (DCA, LCA, T-LCA) were also reduced at 274 this time (Figure 4c). Interestingly, the proportion of the hydrophobic cytotoxic bile acids, 275 DCA and LCA (Figure 4c; t-test, p<0.001 after FDR adjustment), were significantly decreased 276 in the faeces of AOM-treated mice at week 8.

277

The total amount of bile acids in the faeces, as well as the levels of DCA and LCA remained significantly reduced at week 12 (Figures 4a and d; t-test, p<0.001 after FDR adjustment). This agrees with previous work which demonstrated a similar pattern for LCA and DCA in a colitis-associated model of colon cancer ²⁹. Moreover, faecal waters from patients with colon cancer had decreased levels of DCA, LCA and cholate relative to healthy controls ³⁰.

283

The concentrations of primary unconjugated (CDCA, β -MCA) and conjugated (T-CA, T- β -MCA) faecal bile acids were increased in AOM-treated mice at week 24, of which β -MCA was the most abundant (Figure 4e; t-test, p<0.05 after FDR adjustment). There was also an increase in both taurine-conjugated primary and secondary bile acids. In contrast, LCA was significantly reduced (Figure 4e; t-test, p<0.05 after FDR adjustment). Moreover, expression of *ASBT* was significantly reduced (t-test, p<0.01). By week 48, there was no significant change in the bile acid profile (Figure 4a and 4f).

291

292 Colonic inflammatory response to AOM

293 Given that inflammation is a risk factor for colon cancer and can influence colon 294 carcinogenesis, we measured the transcription of several cytokines and chemokines in the 295 distal large intestine (Figure 5a). Significant increases in cytokine and chemokine expression 296 patterns become apparent at week 12, with the expression of $TNF\alpha$, *IL-16*, *IL-12*, *CXCL1*, 297 CXCL2 and CXCL5 significantly up-regulated in AOM-treated mice (Figure 5; t-test, p<0.05, 298 p<0.01, p<0.001 after FDR correction). TNF α remained elevated at week 24, together with 299 an increase in gene expression of IL-6, IL-18 and IL-12 (Figure 5a; t-test, p<0.05 after FDR 300 correction). However, these alterations in cytokine and chemokine transcription were absent 301 by week 48. Consistent with these findings, human colonic tumour cells display a similar 302 temporal response following stimulation with faecal waters isolated from AOM-treated mice 303 relative to PBS-treated mice at the same time-points. At weeks 12 and 24, CXCL2 and CXCL1 304 were significantly increased in the HT29 cells, respectively. In this human cell line, the 305 changes in immunoregulatory gene expression were also increased following stimulation 306 with faecal water prepared from week 48, but these changes were not significant.

307

308 Correlation analyses between the gut microbiota and immunoinflammatory cytokines and309 bile acid composition

310 Correlation analyses were performed to identify any relationships between the microbiome, 311 cytokine transcription and the bile acid pool. At week 8, Allobaculum negatively correlated 312 with *IL-12* (Spearman, p<0.001, R=-1), *Coriobacteriaceae_uncultured* negatively correlated 313 with CXCL1 and IL-6 (p<0.001, R=-0.97; p<0.001, R=-0.95, respectively) and 314 *Defluviitaleaceae_uncultured* negatively correlated with *IL-1*β (Spearman, p<0.001, R=-0.95). 315 No additional correlations between individual taxa and cytokines were observed at any other 316 time-point in AOM-treated mice. At weeks 12 and 24, we observed significant correlations 317 between bile acids and individual taxa (Supplemental Table 4). With the exception of 318 Parasutterella which positively correlated with T-UDCA (Spearman, p<0.001, R=1), all other 319 correlations were negative (Supplemental Table 4). We also investigated whether any 320 correlations existed between CAGs, inflammatory genes, bile acids, and ASBT expression. 321 CAG2 negatively correlated with DCA at week 24 (Spearman, p<0.001, R=-1). At week 48, 322 CAG4 (Spearman, p<0.001, R=0.952) and CAG5 (Spearman, p<0.01, R=0.81) positively 323 correlated with ASBT expression, whilst CAG8 negatively correlated (Spearman, p<0.05, R=-324 0.857).

325 Discussion

326 Decreased α -diversity in the faecal microbiome has been described as a characteristic feature of CRC ^{7, 31}. However, studies have also reported an increase in α -diversity in patients 327 328 with colon cancer ^{32, 33}. This divergence may be associated with the stage of the disease ³¹. In 329 support of this, our data suggest that carcinogenesis begins with an increase in α -diversity as 330 characterised by an increase in PD and observed species in AOM-treated mice at week 8. This 331 suggests that there are more OTUs present in the AOM group which are further away from 332 each other on the phylogenetic tree. The presence of a small number of highly divergent 333 OTUs would greatly impact PD without having a major impact on richness. When considering 334 which taxa might account for the increase in PD but not richness, we identified multiple taxa 335 which were either present in one treatment group and absent in the other or vice versa at 336 week 8. Most of these OTUs fell within the phyla Bacteroidetes (uncultured), Firmicutes (e.g. 337 Erysipelotrichaceae, Roseburia, Blautia) or Tenericutes (Mollicutes). From week 12 onward, 338 this change in α -diversity was no longer apparent. However, by week 12, a significant up-339 regulation of pro-inflammatory signalling was detected in mice administered AOM. At this 340 time-point, the microbial communities became more similar, and one possible explanation 341 may be that the increase in α -diversity observed early in the tumorigenic process is due to 342 the growth of opportunistic pathogens and is reversed later once the immune response 343 becomes more active.

344

345 In this context, the opportunistic pathogen *Clostridium sensu stricto*, which was decreased 346 at week 8 in our study, and then increased at week 12, occurred at a time where the 347 inflammatory response also significantly changed in our mouse model of colon 348 carcinogenesis. A similar positive relationship between this taxon and inflammation was shown in a mouse model of inflammatory bowel disease (IBD) ³⁴. Other taxa that changed 349 350 over time included Turicibacter, which was decreased at week 8 but increased at week 48 in 351 our model. Whilst little is known about a direct role for this genus in inflammation and 352 colorectal cancer, its abundance was previously found to be decreased in TNFa-deficient mice compared to wildtype mice ³⁵. Other studies have also suggested that this genus 353 354 benefits from a pro-inflammatory environment which is consistent with the absence of an overt inflammatory response in our model at week 8³⁶. Finally, *Marvinbryantia*, with the 355 356 exception of week 12, was significantly decreased over time. This genus has also been shown to be reduced in CRC ³⁷. Moreover, in a rat model of colitis, *Marvinbryantia* was significantly 357 358 increased in response to feeding with resistant starch. This was associated with decreased

tumour multiplicity, increased short chain fatty acid production and reduced proliferation
 and inflammation, suggesting that it may have anti-inflammatory and protective properties
 ³⁸. These data reflect the dynamic interplay between inflammation and the microbiome and
 suggest that temporal changes in the abundance of specific genera may be dependent on
 the host inflammatory response at particular time-points during the course of tumorigenesis.

364

365 Turicibacter, the second most abundant member of CAG5, correlates with the activity of 366 *Slc10a2*, which encodes ASBT and helps recycle bile acids from the small intestine back to the liver ³⁹. Moreover, expression of ASBT is sensitive to changes in the microbiome ⁴⁰. We 367 observed that ASBT expression tended to follow a similar dynamic pattern over time as total 368 369 bile acids, but there was no significant correlation after FDR correction between the 370 expression of this transporter and the bile acids measured. However, of the 12 faecal bile 371 acids measured, eight were significantly increased when expression of ASBT was significantly 372 decreased, in particular conjugated bile acids that have high substrate specificity for this 373 transporter ⁴¹. This in turn could potentially lead to increased luminal concentrations of the 374 farnesoid X receptor (FXR) antagonist, T- β MCA, which is normally transported by ASBT, and 375 this, coupled with an increase in the ASBT-independent transport of the FXR agonist, CDCA, 376 may potentially influence tumorigenesis through alterations in FXR signalling.

377

378 The presence of high levels of bile acids also suppresses the activity of Turicibacter ³⁹. 379 Moreover, studies examining the effect of *Turicibacter sanguinis* on bile acid metabolism and 380 transformation suggest that this human isolate can de-conjugate T-CA and transform CA and CDCA by the action of the microbial bile acid-metabolising enzyme 7α -hydroxysteroid 381 dehydrogenase ³⁹. Therefore, fluctuations in the abundance of this taxon over time might 382 383 not only affect bile acid metabolism, but the bile acid profile at any given time-point could 384 also influence the abundance of *Turicibacter*. The next most abundant members of CAG5 are 385 Parasutterella and Bifidobacterium. Of these, Bifidobacterium display sensitivity to 386 fluctuations in bile acids, in particular to the toxic effects of the secondary bile acids such as 387 DCA ⁴². Moreover, colonisation with *Parasutterella* modified the bile acid metabolites, thereby impacting bile acid transport and synthesis⁴³. Taken together, these findings suggest 388 389 that significant changes in the relative abundance of individual taxa responsible for bile acid 390 metabolism and modification may not necessarily be reflected in the expected faecal bile 391 acid profile, or vice versa, particularly in the context of a more complex ecosystem in which members not only metabolise bile acids but are also sensitive to their growth inhibitoryeffects.

394

395 The only correlation between CAGs and bile acids was between the secondary bile acid, DCA 396 and CAG2 and this occurred at week 24. DCA also significantly correlated with Bacteriodes at 397 this time-point. DCA has been shown to be toxic and inhibit the growth of Bacteriodes 44. 398 However, we observed no significant difference in either this taxon or in the faecal levels of 399 DCA at this time-point. This taxon is also the most abundant member of CAG2, which, in turn, 400 was also negatively associated with DCA. Whilst the relative abundance of the taxon within 401 the CAG did not change, another member of this CAG, Bacteroidales S24-7, showed a five-402 fold reduction in AOM-treated mice relative to control mice. Moreover, this taxon was 403 significantly less abundant in AOM-treated mice at this time-point (see Figure 3). 404 Bacteroidales S24-7 has previously been shown to positively correlate with caecal levels of T-DCA in a mouse model of liver regeneration ⁴⁵. Of note, levels of *Bacteroidales* have been 405 406 shown to be significantly reduced in patients with CRC relative to healthy controls ⁴⁶.

407

408 Expression of the bile acid transporter ASBT positively correlated with CAG5 at week 48. At 409 this time-point the abundance of *Bifidobacterium* also significantly increased in AOM-treated 410 mice (see Figure 3). Moreover, within CAG5, the abundance of this taxon increased threefold. Bifidobacteria have bile acid-deconjugating activity which is consistent with the 411 412 normalisation of conjugated bile acids at week 48, relative to week 24, when the abundance of this species significantly increased. Notably, expression of the bile acid receptor FXR is 413 down-regulated in human colorectal tumours and colon cancer cell lines ⁴⁷. Moreover, 414 administration of tauro-conjugated β-MCA, which is an FXR antagonist, accelerated tumour 415 growth and increased the serum levels of pro-inflammatory cytokines in APC^{MIN} mice ⁴⁸. 416 417 Given the ability of bifidobacteria, which are significantly more abundant at week 48, to 418 deconjugate the endogenous FXR antagonist T- β -MCA and relieve its FXR antagonism in 419 mice, this is consistent with a possibly pro-tumorigenic effect of T- β -MCA and FXR 24 weeks 420 after AOM administration.

421

422 CAG4 also positively correlated with ASBT expression at week 48. Although the most 423 abundant taxon within CAG4 was *Bacteroidales S24- 7*, this was comparable within the CAG 424 for PBS- and AOM-treated mice. However, *Akkermansia*, the second most abundant taxon 425 within this CAG, differed between treatments both at the taxon level (see Figure 3) and 426 within CAG4 (approx. 2.5-fold reduction in AOM treated mice). A. muciniphila is one of the most studied species of this genus and displays sensitivity to several bile salts ⁴⁹, further 427 428 highlighting the complexity and inter-relationship between bacterial metabolites and the 429 composition of the microbiome. We also observed a negative correlation between the 430 phylum to which this genus belongs, Verrucomicrobia and the genus itself at weeks 12 and 431 24 with UDCA, and T-CA and T-UDCA respectively. Studies have demonstrated that these 432 bile acids do not affect the growth of *A. muciniphila* ⁴⁹, suggesting that this relationship may 433 be driven by other members of this phylum.

434

435 In contrast, CAG8 negatively correlated with ASBT expression at week 48. The most abundant 436 members of CAG8 are Blautia, Ruminococcaceae_uncultured and Lachnospiraceae, which have all been associated with secondary bile acids and bile acid deconjugation ^{50, 51}. Of these 437 438 three taxa, only the abundance of Lachnospiraceae in CAG8 was differentially altered 439 between PBS- and AOM-treated mice (1.5-fold reduction). Moreover, members of the Lachnospiraceae family negatively correlated with CDCA in IBD ⁵². However, in a model of 440 441 liver regeneration no relationship between either individual caecal bile acids or ASBT was observed with Lachnospiraceae⁴⁵. Despite the bile acid metabolising activity of this taxon, 442 443 we did not observe any significant relationship between Lachnospiraceae and individual bile 444 acids. Moreover, the decrease in abundance is consistent with observations that this taxon is significantly reduced in the gut of individuals with CRC ⁴⁶. Given that ASBT expression and 445 faecal bile acid profile had normalised at week 48 in our study, the role of this CAG in bile 446 447 acid transport and metabolism respectively is unclear.

448

449 Of the genera that correlated with inflammatory gene expression, these occurred at week 8 450 when there was no obvious change in inflammation. Of note, Allobaculum negatively 451 correlated with the expression of IL-12. Little is known about Allobaculum, although studies have shown that it is increased in IBD ⁵³ and with Th 17 cell activity ⁵⁴, suggesting that this 452 taxon may be pro-inflammatory. IL-6, IL-1 β , and CXCL1 have all been linked to colon 453 454 carcinogenesis and at week 8, both IL-6 and CXCL1 negatively correlated with 455 Coriobacteriaceae, whilst Defluviitaleaceae negatively correlated with $IL-1\beta$. Despite these 456 correlations, however, the only significant alterations in inflammatory gene expression 457 occurred at weeks 12 and 24. Previous studies have proposed that particular bacterial clusters or CAGs may be more important in colon tumorigenesis than individual taxa³. It 458 could be argued in our study that CAG1 is pathogenic, given that it contains Citrobacter 55, 459

Hydrogenoanaerobacterium ⁵⁶, and Anaeroplasma ⁵⁷ which have been associated with colon
 cancer. However, the most abundant member of this CAG belongs to the uncultured
 Clostridium vadinBB60 group, and therefore we could not definitively classify this CAG as
 pathogenic or pro-inflammatory.

464

465 Although we observed significant changes in both inflammatory factors and bile acids very 466 early on in the tumorigenic process, few correlations were detected between these factors. 467 However, there are other microbial drivers that could influence colonic tumorigenesis. For instance, gut-microbiota-derived metabolites such as hydrogen sulphide and N-nitroso 468 compounds have been implicated in CRC ⁵⁸. Moreover, oxidative stress and reactive oxygen 469 species have also been linked to CRC ⁵⁹. Indeed, continuous exposure of intestinal epithelial 470 cells to high concentrations of secondary bile acids has been shown to induce the production 471 472 of reactive oxygen species and active nitrogen species ⁶⁰. However, given that we observed 473 a significant decrease in DCA and LCA early in response to AOM, we can only speculate on 474 the contribution of bile acid-induced ROS generation early in the tumorigenic process.

475

476 Early changes in the microbiome or microbiome-associated metabolites could potentially 477 represent early biomarkers for CRC development. A recent human study has examined the 478 microbiome of ACF and ACF with synchronous polyps which likely reflects some of the earliest changes in the microbiome ¹¹. When these human samples were stratified by the 479 presence of ACF alone versus those with ACF and polyp, two distinct microbial clusters were 480 apparent, with compositional changes in Firmicutes predominating ¹¹. Of the significantly 481 changed phyla at weeks 24 and 48 in our study, the majority also occurred within the 482 483 Firmicutes phylum. Furthermore, in taxon-based analysis, the microbiota profile from 484 patients with conventional adenomas was depleted in a network of Clostridia OTUs from families Ruminococcaceae, Clostridiaceae, and Lachnospiraceae⁶¹. Whilst we also saw 485 486 significant changes in Clostridia OTUs from these families, these were divergent, with increases and decreases in abundance seen. Our study identifies early changes in the 487 488 microbiome prior to tumour development and likely reflects the equivalent premalignant 489 lesions (ACF and adenoma) in human studies.

490

491 Most of the significant changes in phyla occurred at week 48 and was characterised by a
492 significant reduction in Verrucomicrobia and an increase in Actinobacteria. This reduction in
493 Verrucomicrobia, if sustained, could be associated with improved outcome as a decrease in

494 this phylum is associated with a reduction in tumour development, invasiveness, and inflammation in a mouse model of colon cancer ^{62, 63}. However, the alterations in 495 496 Actinobacteria observed in our study do not appear to be reflected in human disease and 497 therefore Actinobacteria is unlikely to reflect a pre-cancerous biomarker for CRC in human studies ^{64, 65}. Moreover, bile acids were also investigated for their potential as microbiome-498 associated biomarkers for the development of CRC ⁶⁶, but the findings were unclear. Discrete 499 500 taxonomic changes were also observed in our study which may reflect potential beneficial 501 pre-malignant microbiome biomarker. The composition of the microbiome has also been 502 implicated in treatment response. For example, Vibrio and Psychrobacter, both of which are 503 Gammaproteobacteria, were significantly reduced at all four time points in our study. 504 Notably the presence of intra-tumoral Gammaproteobacteria in pancreatic cancer resulted 505 in treatment resistance to the chemotherapeutic, gemcitabine ⁶⁷. Whether the changes in 506 the faecal microbiome represent prognostic or predictive biomarkers for disease for 507 treatment response warrants further investigation in CRC.

508

509 In conclusion, the first changes we observed in response to AOM treatment were microbial 510 in nature, potentially pro-tumorigenic, and preceded inflammatory changes in the host. 511 Concurrent alterations in the bile acid pool, possibly reflecting a reduction in microbial bile 512 acid metabolism, were also significant in the earlier phase following AOM treatment. Whilst 513 a significant cytokine response ensued, this was largely ameliorated by week 48 when 514 macroscopic adenomas appeared. Our study highlights the complexity of microbe-host 515 interactions in the pathogenesis of colon cancer and the discrete events which occur 516 following a genotoxic insult. Improved understanding of these interactions could lead to 517 better interventional strategies to suppress the development of colon cancer at key stages 518 in the tumorigenic process.

519

520 Additional Information

521 Acknowledgements

522 We acknowledge Pat Casey for his assistance with the animal studies. Graphical abstract was

523 created with BioRender.com.

524

525 Data Availability Statement

The data underlying this article will be shared on a reasonable request to the correspondingauthor.

528 Authors' contributions

- 529 JMK acquired data, and played an important role in interpreting the results and drafted the
- 530 manuscript. CJW, PC and KB acquired data. SM helped to design the work that led to the
- submission. PDC helped draft the manuscript, acquired data, and/or played an important
- role in interpreting the results. SAJ, CGMG, AH and NPH conceived and designed the work
- that led to the submission, played an important role in interpreting the results, and drafted
- the manuscript. All authors approved the final version and agreed to be accountable for allaspects of the work.

536 Ethics approval

- 537 Animal experiments were conducted in accordance with the regulations and guidelines of
- the Irish Department of Health following approval by the University College Cork Animal
- 539 Experimentation Ethics Committee (2011/023).

540

541 Data availability

- 542 The datasets generated and/or analysed during the current study are available from the
- 543 corresponding author on reasonable request.

544

545 **Competing interests**

- 546 The authors are not aware of any competing interests that might be perceived as affecting
- 547 the findings of this study.

548 Funding information

- 549 This work was supported by the APC Innovation Platform. APC Microbiome Ireland is a
- 550 research institute funded by Science Foundation Ireland (SFI) through the Irish Governments
- 551 National Development Plan (Grant SFI/12/RC/2273).

553 References

554 1. Keum N, Giovannucci E. Global burden of colorectal cancer: emerging trends, risk 555 factors and prevention strategies. Nat Rev Gastroenterol Hepatol. 2019;16(12):713-32. 556 Yamagishi H, Kuroda H, Imai Y, Hiraishi H. Molecular pathogenesis of sporadic 2. 557 colorectal cancers. Chin J Cancer. 2016;35:4. Flemer B, Lynch DB, Brown JM, Jeffery IB, Ryan FJ, Claesson MJ, et al. Tumour-558 3. 559 associated and non-tumour-associated microbiota in colorectal cancer. Gut. 2017;66(4):633-43. 560 4. Scott AJ, Alexander JL, Merrifield CA, Cunningham D, Jobin C, Brown R, et al. 561 562 International Cancer Microbiome Consortium consensus statement on the role of the human 563 microbiome in carcinogenesis. Gut. 2019;68(9):1624-32. 564 5. Dulal S, Keku TO. Gut microbiome and colorectal adenomas. Cancer J. 565 2014;20(3):225-31. 566 6. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity 567 of the human intestinal microbial flora. Science. 2005;308(5728):1635-8. 568 Ahn J, Sinha R, Pei Z, Dominianni C, Wu J, Shi J, et al. Human gut microbiome and risk 7. 569 for colorectal cancer. Journal of the National Cancer Institute. 2013;105(24):1907-11. 570 Mangifesta M, Mancabelli L, Milani C, Gaiani F, de'Angelis N, de'Angelis GL, et al. 8. 571 Mucosal microbiota of intestinal polyps reveals putative biomarkers of colorectal cancer. Sci Rep. 2018;8(1):13974. 572 573 Ohigashi S, Sudo K, Kobayashi D, Takahashi O, Takahashi T, Asahara T, et al. Changes 9. 574 of the intestinal microbiota, short chain fatty acids, and fecal pH in patients with colorectal 575 cancer. Dig Dis Sci. 2013;58(6):1717-26. Alrawi SJ, Schiff M, Carroll RE, Dayton M, Gibbs JF, Kulavlat M, et al. Aberrant crypt 576 10. 577 foci. Anticancer Res. 2006;26(1a):107-19. 578 Hong BY, Ideta T, Lemos BS, Igarashi Y, Tan Y, DiSiena M, et al. Characterization of 11. 579 Mucosal Dysbiosis of Early Colonic Neoplasia. NPJ Precis Oncol. 2019;3:29. 580 12. Keane JM, Joyce SA, Gahan CGM, Hyland NP, Houston A. Microbial Metabolites as 581 Molecular Mediators of Host-Microbe Symbiosis in Colorectal Cancer. Results Probl Cell 582 Differ. 2020;69:581-603. 583 13. Ocvirk S, O'Keefe SJ. Influence of Bile Acids on Colorectal Cancer Risk: Potential 584 Mechanisms Mediated by Diet - Gut Microbiota Interactions. Curr Nutr Rep. 2017;6(4):315-585 22. 586 14. Earnest DL, Holubec H, Wali RK, Jolley CS, Bissonette M, Bhattacharyya AK, et al. 587 Chemoprevention of azoxymethane-induced colonic carcinogenesis by supplemental dietary 588 ursodeoxycholic acid. Cancer Res. 1994;54(19):5071-4. 589 15. Adam JK, Odhav B, Bhoola KD. Immune responses in cancer. Pharmacol Ther. 590 2003;99(1):113-32. 591 16. Zuo T, Ng SC. The Gut Microbiota in the Pathogenesis and Therapeutics of 592 Inflammatory Bowel Disease. Front Microbiol. 2018;9:2247-. 593 Arthur JC, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan T-J, et al. 17. 594 Intestinal inflammation targets cancer-inducing activity of the microbiota. Science. 595 2012;338(6103):120-3. 596 Lucas C, Barnich N, Nguyen HTT. Microbiota, Inflammation and Colorectal Cancer. 18. 597 Int J Mol Sci. 2017;18(6):1310. 598 19. Mendes MCS, Paulino DSM, Brambilla SR, Camargo JA, Persinoti GF, Carvalheira JBC. 599 Microbiota modification by probiotic supplementation reduces colitis associated colon 600 cancer in mice. World J Gastroenterol. 2018;24(18):1995-2008. 601 20. Bent R, Moll L, Grabbe S, Bros M. Interleukin-1 Beta-A Friend or Foe in Malignancies? Int J Mol Sci. 2018;19(8). 602

Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory
properties of the cytokine interleukin-6. Biochim Biophys Acta. 2011;1813(5):878-88.

Wang X, Lin Y. Tumor necrosis factor and cancer, buddies or foes? Acta PharmacolSin. 2008;29(11):1275-88.

Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, et al. Gut
microbiota composition correlates with diet and health in the elderly. Nature.
2012;488(7410):178-84.

610 24. Joyce SA, MacSharry J, Casey PG, Kinsella M, Murphy EF, Shanahan F, et al.
611 Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the
612 gut. Proceedings of the National Academy of Sciences. 2014;111(20):7421-6.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time
quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif).
2001;25(4):402-8.

616 26. Rosenberg DW, Giardina C, Tanaka T. Mouse models for the study of colon 617 carcinogenesis. Carcinogenesis. 2009;30(2):183-96.

618 27. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-619 associated microbiota in patients with colorectal cancer. PLoS One. 2012;7(6):e39743.

Fang CY, Chen JS, Hsu BM, Hussain B, Rathod J, Lee KH. Colorectal Cancer StageSpecific Fecal Bacterial Community Fingerprinting of the Taiwanese Population and
Underpinning of Potential Taxonomic Biomarkers. Microorganisms. 2021;9(8).

Liu L, Yang M, Dong W, Liu T, Song X, Gu Y, et al. Gut Dysbiosis and Abnormal Bile
Acid Metabolism in Colitis-Associated Cancer. Gastroenterol Res Pract. 2021;2021:6645970.
Le Gall G, Guttula K, Kellingray L, Tett AJ, Ten Hoopen R, Kemsley EK, et al. Metabolite
quantification of faecal extracts from colorectal cancer patients and healthy controls.
Oncotarget. 2018;9(70):33278-89.

628 31. Ai D, Pan H, Li X, Gao Y, Liu G, Xia LC. Identifying Gut Microbiota Associated With 629 Colorectal Cancer Using a Zero-Inflated Lognormal Model. Front Microbiol. 2019;10(826).

63032.Feng Q, Liang S, Jia H, Stadlmayr A, Tang L, Lan Z, et al. Gut microbiome development631along the colorectal adenoma-carcinoma sequence. Nature Communications. 2015;6:6528.

Scanlan PD, Shanahan F, Clune Y, Collins JK, O'Sullivan GC, O'Riordan M, et al.
Culture-independent analysis of the gut microbiota in colorectal cancer and polyposis.
Environmental microbiology. 2008;10(3):789-98.

34. Zou J, Shen Y, Chen M, Zhang Z, Xiao S, Liu C, et al. Lizhong decoction ameliorates
ulcerative colitis in mice via modulating gut microbiota and its metabolites. Applied
Microbiology and Biotechnology. 2020;104(13):5999-6012.

638 35. Jones-Hall YL, Kozik A, Nakatsu C. Ablation of tumor necrosis factor is associated with
639 decreased inflammation and alterations of the microbiota in a mouse model of inflammatory
640 bowel disease. PLoS One. 2015;10(3):e0119441.

641 36. Rausch P, Steck N, Suwandi A, Seidel JA, Künzel S, Bhullar K, et al. Expression of the
642 Blood-Group-Related Gene B4gaInt2 Alters Susceptibility to Salmonella Infection. PLoS
643 Pathog. 2015;11(7):e1005008-e.

37. Jia W, Rajani C, Xu H, Zheng X. Gut microbiota alterations are distinct for primary
colorectal cancer and hepatocellular carcinoma. Protein Cell. 2021;12(5):374-93.

646 38. Hu Y, Le Leu RK, Christophersen CT, Somashekar R, Conlon MA, Meng XQ, et al.
647 Manipulation of the gut microbiota using resistant starch is associated with protection
648 against colitis-associated colorectal cancer in rats. Carcinogenesis. 2016;37(4):366-75.

649 39. Kemis JH, Linke V, Barrett KL, Boehm FJ, Traeger LL, Keller MP, et al. Genetic
650 determinants of gut microbiota composition and bile acid profiles in mice. PLoS Genet.
651 2019;15(8):e1008073.

40. Zarrinpar A, Chaix A, Xu ZZ, Chang MW, Marotz CA, Saghatelian A, et al. Antibioticinduced microbiome depletion alters metabolic homeostasis by affecting gut signaling and colonic metabolism. Nat Commun. 2018;9(1):2872.

Grosser G, Müller SF, Kirstgen M, Döring B, Geyer J. Substrate Specificities and
Inhibition Pattern of the Solute Carrier Family 10 Members NTCP, ASBT and SOAT. Front Mol
Biosci. 2021;8:689757.

42. Tian Y, Gui W, Koo I, Smith PB, Allman EL, Nichols RG, et al. The microbiome modulating activity of bile acids. Gut microbes. 2020;11(4):979-96.

43. Ju T, Kong JY, Stothard P, Willing BP. Defining the role of Parasutterella, a previously
uncharacterized member of the core gut microbiota. ISME J. 2019;13(6):1520-34.

662 44. Devlin AS, Fischbach MA. A biosynthetic pathway for a prominent class of 663 microbiota-derived bile acids. Nat Chem Biol. 2015;11(9):685-90.

Liu HX, Rocha CS, Dandekar S, Wan YJ. Functional analysis of the relationship
between intestinal microbiota and the expression of hepatic genes and pathways during the
course of liver regeneration. J Hepatol. 2016;64(3):641-50.

46. He T, Cheng X, Xing C. The gut microbial diversity of colon cancer patients and the clinical significance. Bioengineered. 2021;12(1):7046-60.

47. Bailey AM, Zhan L, Maru D, Shureiqi I, Pickering CR, Kiriakova G, et al. FXR silencing
in human colon cancer by DNA methylation and KRAS signaling. Am J Physiol Gastrointest
Liver Physiol. 2014;306(1):G48-G58.

48. Fu T, Coulter S, Yoshihara E, Oh TG, Fang S, Cayabyab F, et al. FXR Regulates Intestinal
673 Cancer Stem Cell Proliferation. Cell. 2019;176(5):1098-112.e18.

49. Hagi T, Geerlings SY, Nijsse B, Belzer C. The effect of bile acids on the growth and
global gene expression profiles in Akkermansia muciniphila. Applied Microbiology and
Biotechnology. 2020;104(24):10641-53.

677 50. Ovadia C, Perdones-Montero A, Spagou K, Smith A, Sarafian MH, Gomez-Romero M,
678 et al. Enhanced Microbial Bile Acid Deconjugation and Impaired Ileal Uptake in Pregnancy
679 Repress Intestinal Regulation of Bile Acid Synthesis. Hepatology. 2019;70(1):276-93.

51. Theriot CM, Bowman AA, Young VB. Antibiotic-Induced Alterations of the Gut
Microbiota Alter Secondary Bile Acid Production and Allow for Clostridium difficile Spore
Germination and Outgrowth in the Large Intestine. mSphere. 2016;1(1).

683 52. Wei W, Wang H-F, Zhang Y, Zhang Y-L, Niu B-Y, Yao S-K. Altered metabolism of bile 684 acids correlates with clinical parameters and the gut microbiota in patients with diarrhea-685 predominant irritable bowel syndrome. World J Gastroenterol. 2020;26(45):7153-72.

53. Palm NW, de Zoete MR, Cullen TW, Barry NA, Stefanowski J, Hao L, et al.
Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. Cell.
2014;158(5):1000-10.

54. Miyauchi E, Kim SW, Suda W, Kawasumi M, Onawa S, Taguchi-Atarashi N, et al. Gut
microorganisms act together to exacerbate inflammation in spinal cords. Nature.
2020;585(7823):102-6.

55. Umar S. Citrobacter Infection and Wnt signaling. Curr Colorectal Cancer Rep.2012;8(4).

694 56. da Silva Duarte V, Dos Santos Cruz BC, Tarrah A, Sousa Dias R, de Paula Dias Moreira
695 L, Lemos Junior WJF, et al. Chemoprevention of DMH-Induced Early Colon Carcinogenesis in
696 Male BALB/c Mice by Administration of Lactobacillus Paracasei DTA81. Microorganisms.
697 2020;8(12).

57. Zeng H, Ishaq SL, Liu Z, Bukowski MR. Colonic aberrant crypt formation accompanies
an increase of opportunistic pathogenic bacteria in C57BL/6 mice fed a high-fat diet. J Nutr
Biochem. 2018;54:18-27.

701 58. Zhang W, An Y, Qin X, Wu X, Wang X, Hou H, et al. Gut Microbiota-Derived
702 Metabolites in Colorectal Cancer: The Bad and the Challenges. Front Oncol. 2021;11:739648.

703 59. Wang X, Huycke MM. Extracellular superoxide production by Enterococcus faecalis
704 promotes chromosomal instability in mammalian cells. Gastroenterology. 2007;132(2):551705 61.

60. Liu Y, Zhang S, Zhou W, Hu D, Xu H, Ji G. Secondary Bile Acids and Tumorigenesis in
Colorectal Cancer. Front Oncol. 2022;12:813745.

Peters BA, Dominianni C, Shapiro JA, Church TR, Wu J, Miller G, et al. The gut
microbiota in conventional and serrated precursors of colorectal cancer. Microbiome.
2016;4(1):69.

Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, et al.
Wild Mouse Gut Microbiota Promotes Host Fitness and Improves Disease Resistance. Cell.
2017;171(5):1015-28.e13.

63. Leystra AA, Clapper ML. Gut Microbiota Influences Experimental Outcomes in Mouse
Models of Colorectal Cancer. Genes (Basel). 2019;10(11).

64. Wu Y, Jiao N, Zhu R, Zhang Y, Wu D, Wang AJ, et al. Identification of microbial markers
across populations in early detection of colorectal cancer. Nat Commun. 2021;12(1):3063.

718 65. Zhang YK, Zhang Q, Wang YL, Zhang WY, Hu HQ, Wu HY, et al. A Comparison Study

719 of Age and Colorectal Cancer-Related Gut Bacteria. Front Cell Infect Microbiol.720 2021;11:606490.

66. Lavelle A, Nancey S, Reimund JM, Laharie D, Marteau P, Treton X, et al. Fecal
microbiota and bile acids in IBD patients undergoing screening for colorectal cancer. Gut
microbes. 2022;14(1):2078620.

67. Geller LT, Barzily-Rokni M, Danino T, Jonas OH, Shental N, Nejman D, et al. Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug

726 gemcitabine. Science. 2017;357(6356):1156-60.

727

728

730 Figure Legends

Figure 1. Alpha (α) and beta (β) diversity are altered across time in mice treated with either PBS or AOM. Faecal samples were collected throughout the experiment and analysed by 16S rRNA gene sequencing 8-, 12-, 24-, and 48-weeks following administration of either PBS or AOM. α-diversity was measured using Shannon, Observed Species and Phylogenetic Diversity (PD) *p<0.05 after FDR correction. Data are presented as median (IQR) (a). PERMANOVA of unweighted Unifrac distances were used to examine the β-diversity visualised by principal coordinate analysis (b). n=7-8 per group.

738 Figure 2. Histograms of the community composition of gut microbiota at the phylum level 739 and co-abundance groups (CAG). The impact of AOM on the major phyla in faecal samples 740 of mice at 8-, 12-, 24-, and 48-weeks following administration of either PBS or AOM (a). Each 741 bar chart represents the average reads of the group (n=7–8). Each phylum is expressed as a 742 percentage of the total number of reads for the particular group. Species with a relative 743 abundance of less than 1% were classified as unassigned. Bar charts showing the proportion 744 of specific CAGs detected in AOM and PBS treated groups (b). Seven CAGs were identified 745 and PERMANOVA determined that all CAGs were significantly different (p<0.05 after FDR 746 adjustment). n=7-8.

Figure 3. Taxa which differed significantly in their abundance between groups. From the data acquired by 16S RNA gene sequencing, operational taxonomic units were clustered based on 97% sequence similarity and taxonomy was assigned using BLAST against the SILVA SSURef data base. Only significantly different taxa are presented here. Data are presented as the z-scores of the abundances scaled by row. Taxa highlighted in red font represent taxa that were altered at two or more time-points. n=7-8 per group. *p<0.05, **p<0.01, ***p<0.001 after FDR adjustment.

Figure 4. Alterations in faecal bile acid profiles and transporter gene expression between AOM and PBS treated mice. Bile acids (a, c-f) were measured by UPLC-MS from mice at 8-, 12-, 24-, and 48-weeks and ASBT (b) was measured by qRT-PCR following administration of either PBS or AOM. Faecal bile acid levels are presented as absolute values. Data are presented as mean ± SEM. n=7-8 per group. *p<0.05, **p<0.01, ***p<0.001.

Figure 5. Alterations in immunoregulatory gene expression between AOM and PBS treated mice. Cytokines and chemokines were measured by qRT-PCR from mice at 8-, 12-, 24-, and 48-weeks following administration of either PBS or AOM. n=7-8 except for samples where the values were below the detection threshold (a). HT29 cells were stimulated for four hours with faecal waters derived from PBS- or AOM-treated mice (1:10 dilution) and changes in

- gene expression measured by qRT-PCR (n=3) (b). The heat maps depict fold change in gene
- 765 expression. *p<0.05, **p<0.01, ***p<0.001 after FDR correction. White box in panel (a)
- 766 depicts gene expression that was greater than the fold change range for the other genes but
- 767 was not significantly different between treatment groups.



b. Beta Diversity





- Proteobacteria
- Cyanobacteria
- Candidate_division_TM7
- Actinobacteria
- Deinococcus_Thermus
- Deferribacteres
- Tenericutes
- Verrucomicrobia
- Bacteroidetes
- Firmicutes





d. week 48

c. week 24





-2

0 Row Z-Score

a. Total bile acids

b. Colonic ASBT gene expression











÷ Ŧ

Τ-βΜCΑ

¥. 🛉

T-UDCA

÷







Confirmation of Publication and Licensing Rights

November 18th, 2022 Science Suite Inc.

Subscription:			
Agreement number:			
Journal name:			

Individual DB24NUZQPD British Journal of Cancer

To whom this may concern,

This document is to confirm that Aileen Houston has been granted a license to use the BioRender content, including icons, templates and other original artwork, appearing in the attached completed graphic pursuant to BioRender's <u>Academic License Terms</u>. This license permits BioRender content to be sublicensed for use in journal publications.

All rights and ownership of BioRender content are reserved by BioRender. All completed graphics must be accompanied by the following citation: "Created with BioRender.com".

BioRender content included in the completed graphic is not licensed for any commercial uses beyond publication in a journal. For any commercial use of this figure, users may, if allowed, recreate it in BioRender under an Industry BioRender Plan.



For any questions regarding this document, or other questions about publishing with BioRender refer to our <u>BioRender Publication Guide</u>, or contact BioRender Support at <u>support@biorender.com</u>.