Gastrointestinal Microflora and Mucins May Play a Critical Role in the Development of 5-Fluorouracil-Induced Gastrointestinal Mucositis

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5-Fluorouracil (5-FU) is a commonly used chemotherapy agent in clinical oncology practice. Two of its major side effects are mucositis and diarrhoea. The structure of mucins offers mucosal protection, and allows maintenance of intestinal flora by providing attachment sites and preventing bacterial overgrowth and/or penetration. The aim of this study was to investigate changes in mucin secretion and microflora following treatment with 5-FU. Female DA rats were given a single 150 mg/kg i.p. dose of 5-FU. Rats were killed at various time points after treatment. Control rats received no treatment. Jejunum, colon and faecal samples were collected. Standard microbiological culture techniques were used to identify bacteria, and real-time PCR was used to quantify bacteria in faecal samples. Goblet cells and cavitated goblet cells (having undergone mucus exocytosis) were also counted. Statistical analysis was carried out using Kruskal-Wallis test, a non-parametric method of testing equality of group medians. Following treatment with 5-FU, we showed decreases in *Clostridium spp.*, *Lactobacillus spp.* and *Streptococcus spp.*, and an increase in *Escherichia spp.* in the jejunum. In the colon, 5-FU caused decreases in *Enterococcus spp.*, *Lactobacillus spp.* and *Streptococcus spp.* Real-time PCR of faecal samples showed decreasing trends in *Lactobacillus spp.* and *Bacteroides spp.*, and an increasing trend in *E. coli*. Significant increases ($P < 0.05$) were seen in *Clostridium spp.* and *Staphylococcus spp.* at 24 h. Goblet cell numbers decreased significantly in the jejunum from 24–72 h, with a significant increase in the percentage of cavitated goblet cells. In conclusion, 5-FU treatment causes significant changes in intestinal flora and mucin secretion in rats. These changes could result in systemic effects and, in particular, may contribute to the development of chemotherapy-induced mucositis. Exp Biol Med 234:430–441, 2009

Key words: mucositis; chemotherapy; intestine; microflora

Introduction

Mucositis is a major oncological problem, caused by the cytotoxic effects of cancer chemotherapy and radiotherapy. Approximately 40% of patients receiving standard dose chemotherapy and 100% of patients receiving high dose chemotherapy and stem cell or bone marrow transplantation exhibit the pain, ulceration, bloating, vomiting and diarrhoea associated with mucositis (1–3). 5-Fluorouracil (5-FU) is one of the most commonly used chemotherapy agents in clinical oncology practice and is associated with significant mucositis (4).

Previous studies have indicated that gastrointestinal microflora may be involved in the development of chemotherapy-induced mucositis and diarrhoea (5, 6). The microflora of the gastrointestinal tract (GIT) is a highly complex

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ecosystem consisting of both aerobic and anaerobic bacteria (7–9). Gastrointestinal microflora have a number of key functions including epithelial protection, and metabolism of, bilirubin, intestinal mucins, pancreatic enzymes, fatty acids, bile acids, cholesterol and steroid hormones (10). Furthermore, gastrointestinal bacteria function to process nutrients, regulate intestinal angiogenesis, and work with the immune system (11, 12). Intestinal flora varies between races, sex, age and diet within humans and there is some variation between humans and other species (13). Diseased states and/or associated therapeutic regimens can alter the composition of the gastrointestinal microflora. Acute diarrhoea causes a profound alteration in bacterial populations of the gut (7). Coliform numbers are increased in the human small intestine, and faecal flora consists of more *Proteus spp.* and *Pseudomonas spp.* (7). The rapid transit of stools through the human colon during diarrhoea results in a decrease in the anaerobic population of the colon. Resolution of diarrhoea is accompanied by the restoration of the normal microflora (7). As previous research has shown that the microflora of the gastrointestinal tract also changes after treatment with irinotecan, another cytotoxic chemotherapy agent known to induce diarrhoea (5), it is highly likely that treatment with 5-FU will also alter the gastrointestinal microflora.

The integrity of the "normal" intestinal flora is maintained by the structure of mucins, by providing attachment sites for intestinal flora and pathogenic bacteria (14), as well as simultaneously protecting the mucosa from bacterial overgrowth and/or penetration (15). The mucins also protect the epithelium from digestion by acting as substrates for the enzymes produced by the intestinal flora, such as α-galactosidase, β-N-acetylgalactosaminidase, sialidase, β-glucuronidase, blood group degrading enzymes and proteases (15). The metabolism of mucin may serve to regulate the microecology of the intestinal luminal environment (15). Acidic mucins have been suggested to protect against the translocation of bacteria (16). Mucus offers a number of ecologic advantages to intestinal bacteria. Bacteria are able to degrade mucus, which provides a direct source of carbohydrates and peptides, and exogenous nutrients (vitamins and minerals) (16). The mucus also contains receptors recognising specific adhesion proteins (17). Bacteria colonising the mucus can also avoid rapid expulsion through the intestine, imparting a growth advantage (16). It is not known whether goblet cell numbers or mucus secretion is increased in response to bacterial mucolysis (16).

Treatment with irinotecan has previously been shown to cause goblet cell hyperplasia with accompanying mucous hypersecretion in the caecum (18). Methotrexate has also been shown to induce goblet cell depletion in the small intestine (19). A recent study of mucins and goblet cells in colitis suggests that the goblet cells and mucins may be regulated by interactions of specific bacterial peptides with the gastrointestinal mucosa (20). Bacterial peptides have been shown to cause interleukin (IL)-8 and mucin secretion by colon epithelial cell lines (20). These results suggest a strong link between bacteria (intestinal flora) and mucin secretion, both of which have been shown to be affected in chemotherapy-induced mucositis (5, 21–23). Therefore, the aim of the present study was to determine if 5-FU-induced mucositis can be correlated with changes to the gastrointestinal microflora, the composition and secretion of mucins in the GIT, and goblet cell numbers and integrity.

Methods

Animals. The animals used in this study were inbred female Dark Agouti (DA) rats, weighing between 150 and 170 g. The rats were single-housed in a regular animal facility at 22 ± 1°C and subject to a 14:10 h light-dark cycle. Approval for the use of animals was granted by the Animal Ethics Committees of the Institute of Medical and Veterinary Science (IMVS) and the University of Adelaide, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (2004). Animals were monitored twice daily for the duration of the study.

Experimental Design. Eighty-one rats were randomly assigned to groups. For each time point, one group of rats ($n = 6$) received 5-FU and one group of control rats ($n = 3$) received no treatment. Administration of 5-FU involved intraperitoneal injection of 150 mg/kg 5-Fluorouracil (Mayne Pharma Ltd, Melbourne, Australia), previously shown to cause gastrointestinal mucositis at this dose (24–26), given as a single dose to allow investigation of the specific effects of 5-FU. Groups of rats were killed via exsanguination and cervical dislocation while under deep 3% halothane in 100% O_2 anaesthesia, at times 30, 60 and 90 min, 2, 6, 12, 24, 48, and 72 h post treatment. Immediately prior to anaesthesia, faecal samples were aseptically collected by directly collecting the excreted faeces immediately as it left the rat into a sterile container in an area cleaned with 70% ethanol. Samples were frozen in liquid N_2 and stored at −70°C until required. Prior to cervical dislocation, a blood sample was collected via cardiac puncture. The GIT (from the pyloric sphincter to the rectum) was dissected out and separated into the small intestine (pyloric sphincter to ileocaecal sphincter) and colon (ascending colon to rectum). The small intestine was flushed with sterile, chilled distilled water. Two 1 cm samples were collected at 25% of the length of the small intestine (jejunum) for culture and histology. The colon was also flushed with sterile, chilled distilled water, and contents were collected for electrolyte analysis. Two 1 cm samples were taken at 50% of the length of the colon for culture and histology. The stomach was dissected from the rat and contents emptied. Two small pieces (1 cm × 0.5 cm) of stomach were collected for culture and histology. All samples collected for culture were stored at −70°C until

required, and those for routine histological examination were fixed in 10% neutral buffered formalin.

Electrolyte Analysis. Blood samples were centrifuged (Hereus, Finland) at 3000 rpm for 5 min. The serum was collected into a fresh tube and analysed by the Department of Clinical Pathology, IMVS, Adelaide, South Australia. Measurements for sodium, potassium, bicarbonate, chloride, anion gap, and measured osmolality were measured from serum samples.

Histological Examination. Fixed samples of stomach, jejunum, and colon were processed and embedded in paraffin. Sections of 4 μm thickness were cut and mounted on glass slides. Routine haematoxylin and eosin (H&E) staining was performed. Sections were examined using light microscopy and reported on by specialist veterinary pathologist (Dr. John Finnie, IMVS).

Alcian Blue-PAS Stain. Sections were dewaxed in xylene and rehydrated through a graded series of alcohols. Sections were stained in Alcian Blue (1% Alcian Blue 8GX (CI 74240) in 3% glacial acetic acid) for 5 min, then rinsed in distilled water. Sections were oxidised in 1% periodic acid before washing in running water and rinsing in distilled water. Sections were treated for 15 min in Schiff's reagent and washed for 7 min in running water. Slides were dehydrated, cleared and mounted.

Quantitative Histology. To determine the effect of 5-FU on mucus secretion, goblet cells were counted. Decreased goblet cells indicated release of mucins from the mucosal surface, and cavitation of mucus cells is a sign of accelerated mucus secretion by compound exocytosis (27). Therefore, both the number of goblet cells and percentage of cavitated cells were analysed, according to a method previously described (27). Briefly, a cavitated cell is recognised by apical indentation into the intracellular store of mucus granules. Goblet cells and cavitated cells in crypts and villi that were deemed to be greater than 80% complete were counted, with a total of at least 15 villi/crypts per section analysed.

Culture of Samples. To determine the flora of the DA rat treated with 5-FU, a variety of selective and non-selective media (Oxoid, Adelaide, Australia) was used in an attempt to identify as many bacteria as possible from the GIT. For detailed methodology please refer to http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17202590. The level of growth of each bacterium was graded using a qualitative assessment technique using the following criteria: 0, no growth; 1, very light growth (less than 10 cfu); 2, light growth (growth in the original inoculation zone only); 3, moderate growth (growth in the first streak line); and 4, heavy growth (growth in the second streak line or greater). All gradings were conducted in a blinded fashion (AMS). This grading system had previously been validated in our laboratory using quality control organisms and sample organisms to ensure consistent results (5).

Bacterial Strains and Culture Conditions. The bacterial strains used as positive and negative controls in this study included *Bacteroides fragilis* ATCC 25285, *Bifidobacterium breve* ATCC 15700, *Clostridium botulinum* ATCC 13124, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 47077, *Lactobacillus acidophilus* ATCC 314 and *Staphylococcus epidermidis* ATCC 12228 (Cryosite, Lane Cove, NSW, Australia). Bacteria were cultured in nutrient broth at 37°C overnight. Bacteria requiring anaerobiosis for growth were cultured at 37°C in and anaerobic jar (Oxoid, Australia) in Cooked Meat broth (Oxoid), Thioglycollate broth (Oxoid) or MRS broth.

Extraction and Purification of DNA from Bacterial Culture and Faecal Samples. *DNA Extraction from Bacteria.* DNA was extracted from known bacterial strains (Table 1) using a DNeasy Tissue Mini Kit (Qiagen). Briefly, bacteria were grown to log phase in nutrient broth. Cells were harvested by centrifuging for 10 min at 5000 × *g*. Gram positive bacteria were lysed in enzymatic lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM sodium EDTA, 1.2% Triton® X-100, 20 mg/mL lysozyme) at 37°C, followed by proteinase K at 70°C. Gram negative bacteria were lysed in provided buffer and proteinase K at 55°C, followed by further lysis in buffer at 70°C. For both gram positive and gram negative bacteria, DNA was bound to the membrane in the provided spin-column. The membrane was washed with 2 buffer solutions before DNA was eluted from the membrane into a centrifuge tube. DNA concentration was determined using a spectrophotometer (Eppendorf, Sydney, Australia) and diluted to a final working concentration of 10 ng/μL.

DNA Extraction from Samples. DNA was extracted from rat faecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Doncaster, Australia). Briefly, a pellet of rat faeces was homogenised in lysis buffer and heated at 70°C. Samples were centrifuged and the supernatant collected in a centrifuge tube. Inhibitors were adsorbed to InhibitEX tablets before samples were centrifuged again and the supernatant collected. Proteins were digested with proteinase K and buffer. DNA was then bound to the membrane in the provided spin column. The membrane was washed with 2 wash buffers before DNA was eluted into a centrifuge tube. DNA concentration was determined using a spectrophotometer, and diluted to a final working concentration of 5 ng/μL.

Real-Time PCR. All primers used in this study have been used previously (Table 1). General optimisation of each set of primers was carried out to determine optimal working conditions in the Rotorgene rotary cycler (Corbett Research, Mortlake, NSW, Australia), using Quantitect SYBR Green Mastermix (Qiagen).

Real-time PCR was carried out on a Corbett Rotorgene rotary cycler (Corbett). Primers used in this study have been used previously (28–31) (Table 1). PCR mixtures consisted of 1× Quantitect® SYBR Green Mastermix (Qiagen), 2.5 ng/μL of each primer and 10 ng of DNA in a volume of 10 μL. The cycling parameters consisted of enzyme activation

Table 1. Primers Used for Real-Time PCR

Primer	Standard	Other reactive organisms	Anneal temp	Extension	Cycles	Product (bp)
Bacteroides spp. (28)	*B. fragilis* ATCC 25285	All *Bacteroides* genus	55°C 15 s	20 s	50	106
Bifidobacterium spp. (30)	*B. breve* ATCC 15700	*Bifidobacterium breve, B. bifidum, B. adolescentis, B. longum, B. minimum, B. angulatum, B. catenulatum, B. pseudocatenulatum, B. dentium, B. ruminatum, B. thermo philum, B. subtile, B. bifidum, B. boum, B. animalis, B. choerinum, B. gallicum, B. pseudolongum, B. magnum, B. infantis, B. indicum, B. gullinarum, B. pullorum, B. saeculare, B. suis*	60°C 50 s	30 s	45	243
Clostridium spp. (30)	*C. botulinum* ATCC 13124	*Clostridium botulinum, C. perfringens, C. homoproprionicam, C. cadaveris, C. intestinalis, C. putrificum, C. novyi, C. sporogenes, C. tyrobutyricum, C. kluyveri, C. ljungdahlii, C. scatologenes, C. acetireducens, C. subterminale, C. esterheticum, C. argentinense, C. chauvoei, C. sardiniensis, C. paraputrificum, C. longisporum, C. septicum, C. cellulovorans, C. barati, C. absonum, C. carnis, C. butyricum, Eubacterium cudayi, E. nitritogens, E. moniliforme, E. multiforme*	50°C 20 s	30 s	45	120
E. coli (29)	*E. coli* ATCC 25922	*Escherichia coli*	57°C 15 s	20 s	45	95
Enterococcus spp. (30)	*E. faecalis* ATCC 47077	*Enterococcus faecalis, E. faecium, E. asini, E. saccharolyticus, E. casseliflavus, E. gallinarum, E. dispar, E. flavescens, E. hirae, E. durans, E. pseudoavium, E. raffinosus, E. avium, E. malodoratus, E. mundtii, E. azikeevi, E. canis, E. gilvus, E. haemoperoxidus, E. hermanniensis, E. moraviensis, E. pallens, E. phoeniculicola, E. villorum, E. rottae*	61°C 15 s	20 s	60	144
Lactobacillus spp. (30)	*L. acidophilus* ATCC 314	*L. acidophilus, L. amylovorus, L. delbruecki, L. amylolyticus, L. acetotolerans, L. crispatus, L. amylophilus, L. johnsonii, L. gasseri, L. fermentum, L. pontis, L. reuteri, L. mucosae, L. vaginalis, L. panis, L. oris, L. pentosus, L. plantarum, L. collinoides, L. alimentarius, L. farciminis, L. brevis, L. buchneri, L. kefiri, L. fructivorans, L. mali, L. animalis, L. murinus, L. ruminis, L. agilis, L. salivarius, L. aviaruis, L. sharpeae, L. manihotivorans, L. rhamnosus, L. casei, L. zeae, L. sakei, Pediococcus pentosaceus, P. parvulus, P. acidilactici, P. dextrinicus, Weissella halotolerans, W. confusus, W. paramesenteroides, W. hellenica, W. viridescens, W. kandleri, W. minor, Leuconostoc lactis*	50°C 20 s	30 s	45	341
Staphylococcus spp. (31)	*S. epidermidis* ATCC 12228	*S. epidermidis, S. aureus, S. saprophyticus, S. cohnii, S. haemolyticus, S. hominis, S. lugdenensis, S. xylosus, S. wareni*	46°C 8 s	20 s	68	462

at 95°C for 10 min, followed by cycles of 95°C for 15 s melting, 15 s annealing (see Table 1 for annealing temperatures) and 72°C at 20 s extension. The SYBR green fluorescent signals were acquired at 72°C. Standard curves were constructed from PCR reactions using 10-fold serial dilutions of known bacterial DNA. Data was analysed using Rotorgene 6.0 software (Corbett Research).

Bacterial Susceptibility. Bacterial susceptibility to 5-FU was determined using a standard antibiotic susceptibility testing method (32). The same standards from real-time PCR experiments were used, and also *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. Briefly, serial dilutions of 5-FU were diluted in sterile water for injection, and 10 µL was put onto blank discs (Oxoid). Sensitest Agar plates were used for *Enterococcus faecalis*, *Escherichia coli*, *Streptococcus pneumoniae, Staphylococcus aureus* and *Staphylococcus epidermidis*. Anaerobic agar (Oxoid) was used for *Bacteroides fragilis*, *Bifidobacterium breve* and *Lactobacillus acidophilus* and *Clostridium botulinum*. Discs were placed onto a lawn culture of each bacterium, incubated overnight at 37°C, and zones of inhibition measured. All experiments were carried out in triplicate.

Statistical Analysis. Statistical analysis was performed using multiple Mann-Whitney *U* tests for non-parametric data, using GraphPad Prism 5.0 software. Differences between group medians were considered significant at $P < 0.05$, and adjusted to correct for Type I error with Bonferroni correction.

Results

Effects of 5-FU on Electrolytes. Electrolyte levels were altered in rats treated with 5-FU (Fig. 1). Serum sodium levels were lower in experimental rats after treatment compared with control rats. Bicarbonate levels were significantly higher 12 h after treatment ($P < 0.005$). The anion gap was significantly lower in experimental rats 12 h ($P < 0.002$). Serum potassium, chloride and measured osmolality levels did not change significantly after treatment (data not shown).

Histological Changes Caused by 5-FU. Pathological changes caused by 5-FU were seen in the stomach, jejunum and colon (Fig. 2). A loose infiltration of eosinophils in the deep lamina propria was seen in the stomach, with occasional necrotic cells in the glandular epithelium 24 h after chemotherapy treatment. No significant changes were seen from 48 h. Mild villous clubbing and fusion, and enterocyte hyperplasia were seen in the jejunum 30 min after chemotherapy. Villous atrophy was apparent 90 min after chemotherapy, with apoptotic bodies evident in the crypts by 6 h. Villous atrophy and apoptotic bodies were present at 12 and 24 h, and by 72 h eosinophils and lymphocytes had infiltrated the lamina propria, with cytoplasmic vacuolation of the enterocytes. The colon exhibited numerous apoptotic bodies in the crypts 12–24 h after treatment.

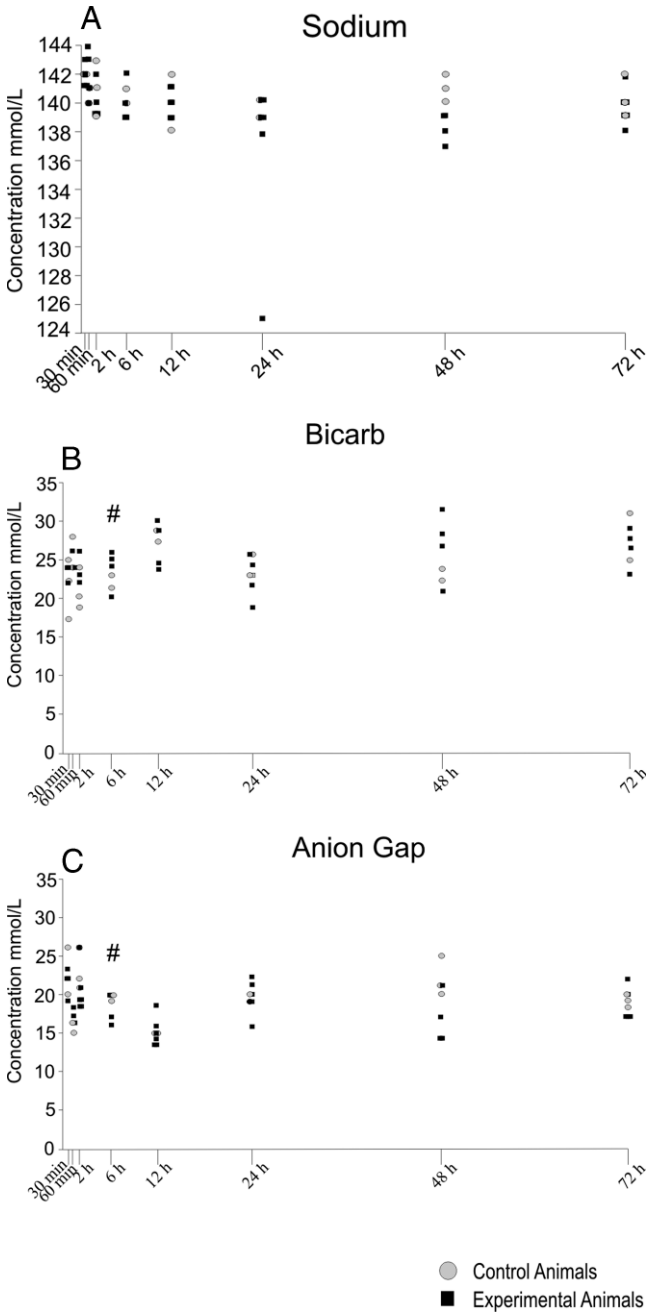

Figure 1. Electrolytes. A) Sodium. Serum sodium levels were lower in experimental rats after treatment compared with control rats. B) Bicarbonate. Bicarbonate levels were significantly higher 12 h after treatment. C) Anion Gap. The anion gap was significantly lower in experimental rats 12 h after treatment. # Denotes significance.

Goblet Cell Composition and Distribution. *Stomach.* No noticeable changes to the mucin composition of the stomach were seen following treatment with 5-FU (data not shown).

Jejunum. In the jejunum, goblet cells became condensed at the base of the villi 30 min after treatment. These goblet cells began to 'move' up the villi until 2 h post treatment. At 24–48 h the goblet cells became enlarged and

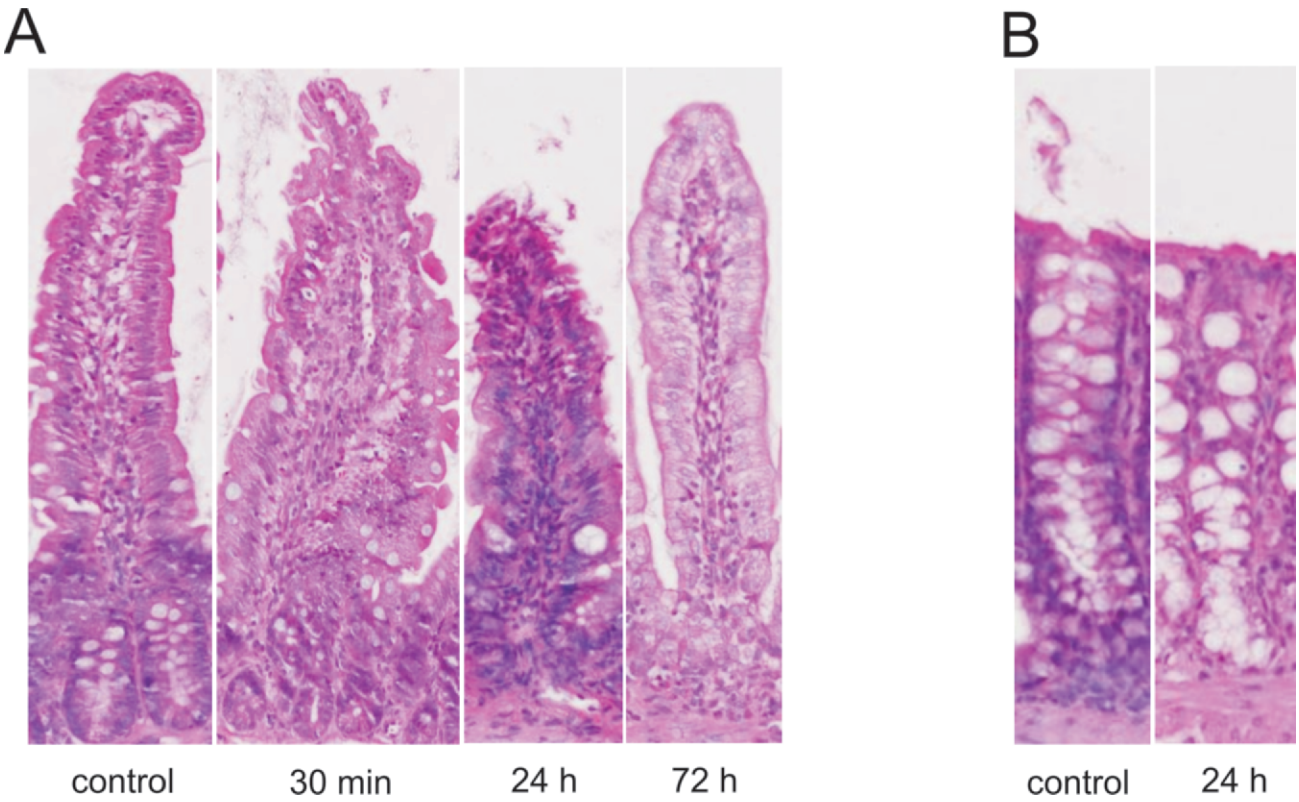

Figure 2. Pathology. A) Histological changes in the jejunum following treatment. B) Histological changes in the colon following treatment.

dilated (Fig. 3). There were no changes in the composition of mucins following 5-FU treatment.

Colon. At 6 h following chemotherapy treatment, mixed mucins were only seen in the base of the crypts. By 12 h, the mucins at the base of the crypt were acidic, and the goblet cells were partially dilated. At 24 h following 5-FU administration, the majority of mucins were mixed (with a few neutral) and the goblet cells were dilated.

Effect of 5-FU on Mucin Discharge. All goblet cells were counted per villus/crypt in the jejunum and colon. Mucus cells in the stomach could not be counted due to the histological structure. Goblet cell counts in untreated rats had 14.25 ± 0.73 (mean ± SEM) goblet cells/villus, with 37.37 ± 2.40% (mean ± SEM) of those deemed to be cavitated. Total goblet cells in the villi were decreased significantly at 72 h compared with control ($P < 0.0006$)

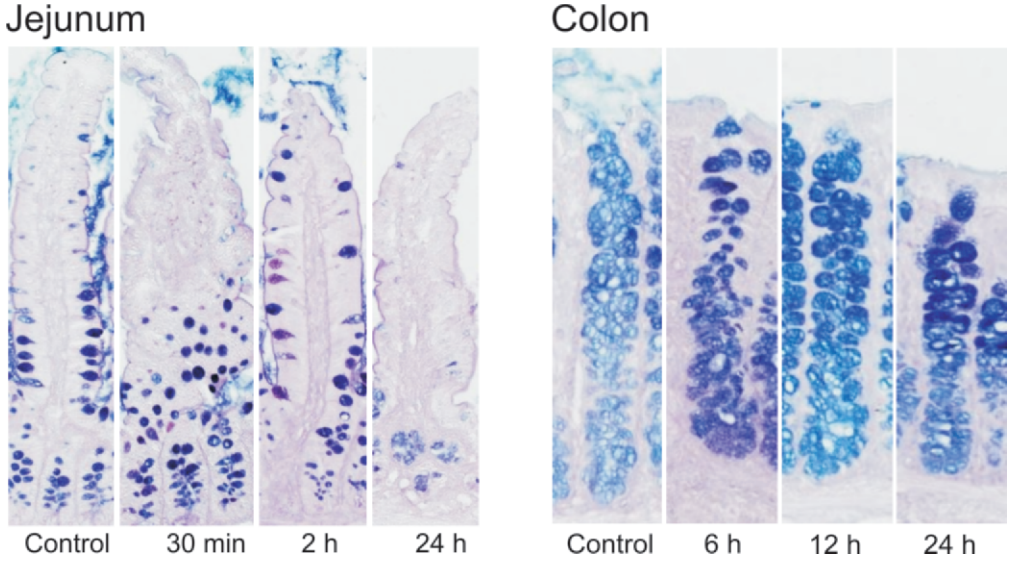

Figure 3. Alcian Blue-PAS Staining in the jejunum and colon, demonstrating changes in mucin composition and distribution following treatment.

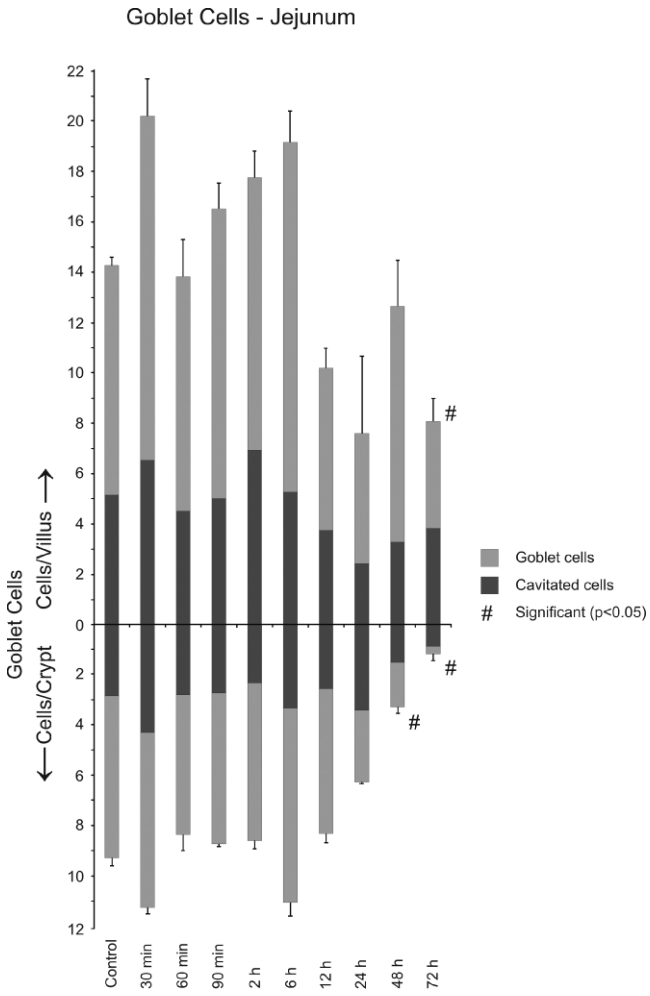

Figure 4. Goblet cells. Goblet cells counted in the jejunum, divided into villi and crypts, and into cavitating goblet cells and intact goblet cells. # Denotes significance.

(Fig. 4). The percentage of cavitated cells appeared to increase at 72 h after treatment, but this was not deemed significant after adjustment using Bonferroni correction. The jejunum crypts exhibited a significant decrease in total goblet cell numbers 48–72 h after treatment ($P < 0.004$). The colon exhibited a general decrease in total goblet cell numbers after treatment with 5-FU, although not significant after performing Bonferroni correction. The percentage of cavitated cells did not change significantly after treatment (not shown).

Culture. Changes were seen in the flora of the stomach, jejunum, colon, and faeces (Fig. 5). The organisms identified were consistent with the expected gastrointestinal microflora of rats. The stomach mucosal surface of rats was found to harbour copious amounts of *Clostridium spp*. and *Lactobacillus spp*., with a moderate amount of *Streptococcus spp*. Changes in levels of bacteria following 5-FU were seen. *Clostridium spp*. (Fig. 5A) and *Escherichia spp*. (not shown) levels decreased at 24 h. *Staphylococcus spp*. (Fig. 5A) levels also decreased, but at 6 h post treatment.

The jejunal mucosal surface was found to accommodate relatively small amounts of organisms compared with the stomach, colon and faeces. The most prominent were *Clostridium spp*., *Lactobacillus spp*. and *Streptococcus spp*. Changes were seen in levels of bacteria after chemotherapy. *Clostridium spp*. decreased from 6–12 h post treatment. *Lactobacillus spp*. decreased from 60 min post treatment, with a larger decrease at 6 h. *Escherichia spp*. peaked at 48–72 h post treatment (Fig. 5B).

The mucosal surface of the colon in control rats was found to accommodate a large amount of organisms. The most prominent was *Lactobacillus spp*., with smaller amounts of *Streptococcus spp*. and *Clostridium spp*. Changes were seen in the levels of bacteria following chemotherapy. There was an increase in *Clostridium spp*. after 24 h (not shown), and *Escherichia spp*. from 2–6 h and again from 48–72 h (Fig. 5C). *Lactobacillus spp*. decreased from 1–2 h and 12–24 h (Fig. 5C). *Streptococcus spp*. fluctuated after chemotherapy (not shown).

In all rats, the bacterial content of the faeces was found to be higher than the colon, jejunum or stomach. The most prominent bacteria were *Clostridium spp*., *Enterococcus spp*., *Eubacterium spp*. and *Lactobacillus spp*. Changes were seen following treatment with 5-FU. *Clostridium spp*. decreased 2 h following treatment. *Escherichia spp*. decreased from 2–24 h post treatment. *Proteus spp*. decreased from 2–6 h post treatment. *Streptococcus spp*. decreased at 72 h.

Real-Time PCR. Differences in *Bifidobacterium spp*., *Bacteroides spp*., *Clostridium spp*., *E. coli*, and *Lactobacillus spp*. were seen in rats treated with 5-FU. However, following Bonferroni correction these were not deemed significant. The following trends were seen: *Bifidobacterium spp*. exhibited fluctuations between time points; *Bacteroides spp*. decreased at 48 h; *Clostridium spp*. increased after treatment; *E. coli* increased at 48 h; and *Staphylococcus spp*. increased at 24 h. *Lactobacillus spp*. decreased from 12–24 h, and *Enterococcus spp*. was decreased from 2–48 h after treatment (Fig. 6).

Bacterial Susceptibility to 5-FU. *E. coli* and *P. aeruginosa* were not susceptible to 5-FU at any concentration. *E. faecalis*, *S. pneumoniae*, *L. acidophilus*, *B. lactis*, *C. botulinum* and *S. epidermidis* showed susceptibility, as summarised in Table 2.

Discussion

5-FU is one of the most commonly used chemotherapy agents used in clinical oncological practice (4). However, side effects are prevalent, and result from the inhibition of rapidly dividing tissues, including bone marrow, haemopoietic cells and gastrointestinal mucosal cells (4). Clinical studies have shown 5-FU to cause mucositis, myelosuppression, nausea, emesis, and diarrhoea, associated with marked histopathological changes (4, 33). However, the association between mucositis and the intestinal bacteria remains unclear. This study has demonstrated changes in

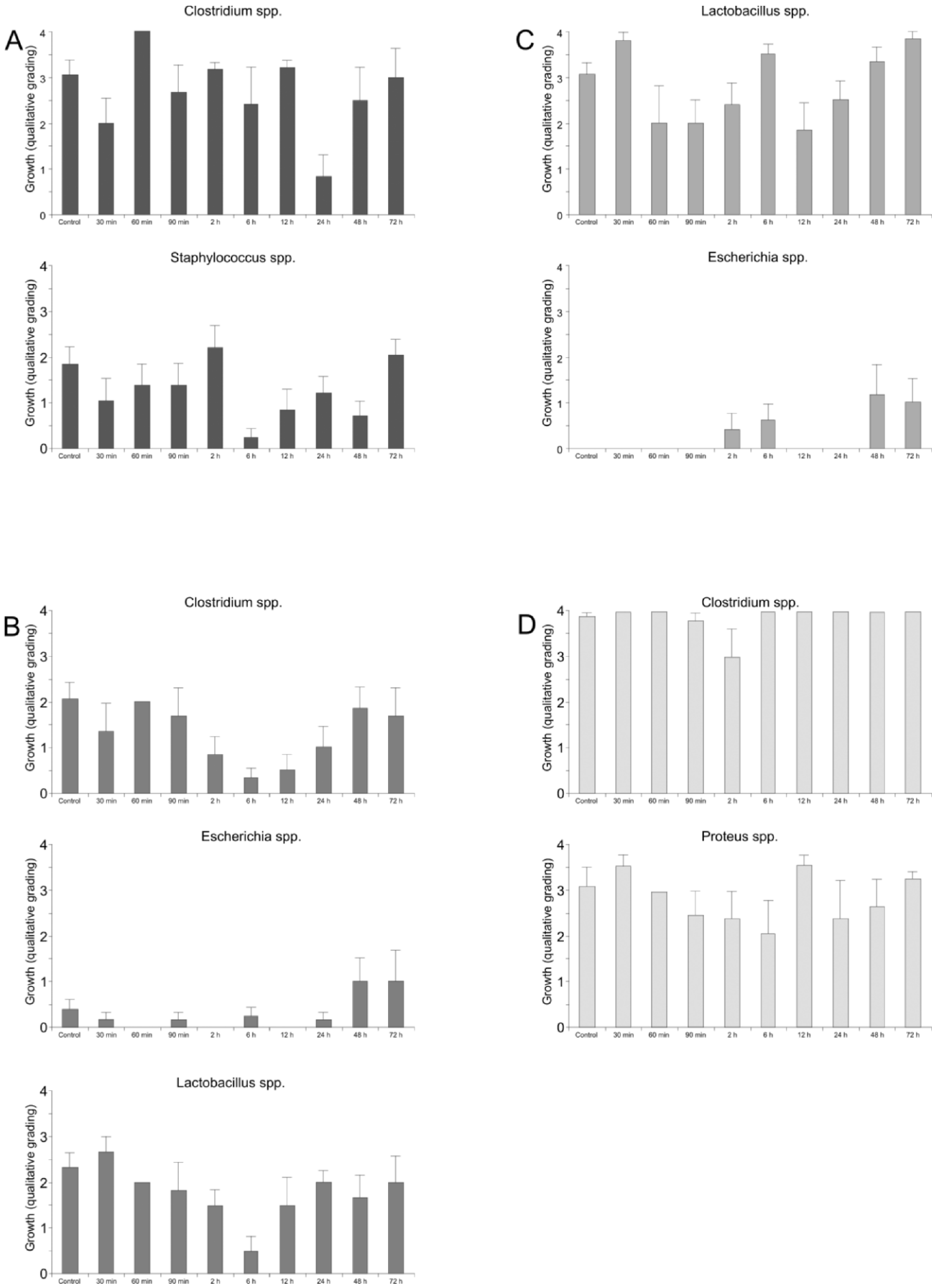

Figure 5. Qualitative microbiology. Changes to microflora in the A) stomach, B) jejunum, C) colon and D) faeces.

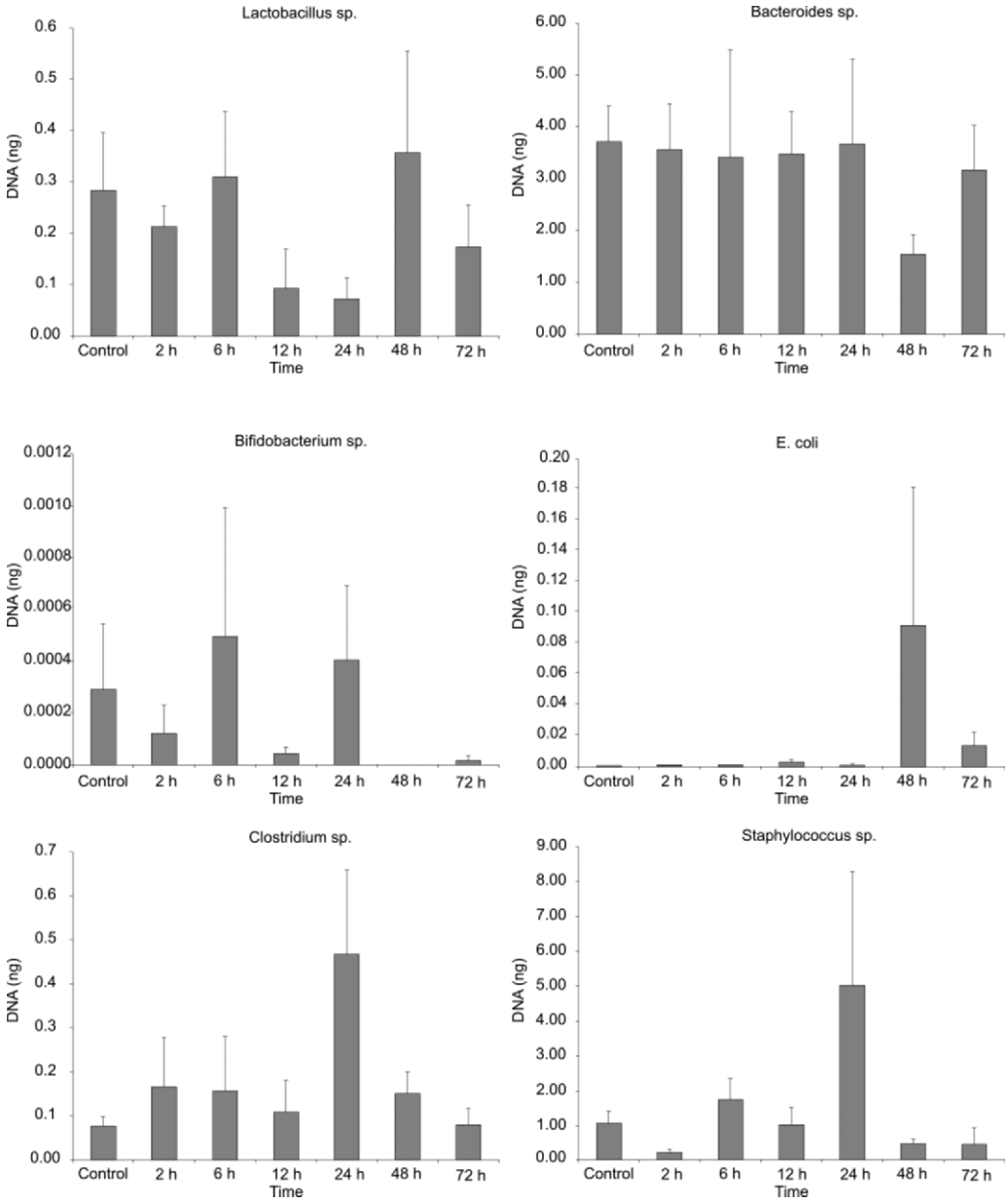

Figure 6. Quantitative PCR. Changes to faecal microflora.

intestinal bacteria and goblet cells which correlate with histological changes at early time points after 5-FU treatment. We have also shown a variety of other changes in electrolytes and correlated them with histological changes, suggesting that these components may play a crucial role in the development of 5-FU-induced mucositis.

Histological evidence of damage is seen in the jejunum as early as 30 min after treatment, with greatest damage seen 24 h after treatment in the stomach, jejunum and colon, and was consistent with previous studies (4, 33). Mucin metabolism has previously been demonstrated to be influenced by multiple doses of 5-FU (34). Our study has shown that a single dose of 5-FU also influences mucins, affecting composition, distribution and secretion. Goblet cells in the jejunum tend to decrease temporally after treatment with 5-FU, with crypt goblet cells being affected the most, decreasing significantly from 24–72 h. Cavitated cells can be counted as a measure of cells secreting mucin, and expressed as a percentage of total goblet cells (35). Cavitated cells in the jejunum crypts increased significantly from 24–72 h. Cavitated cells in the villi did not change significantly, suggesting that mucin secretion in the jejunum as a result of treatment with 5-FU occurs primarily in the crypts. The implications of both decreased goblet cells and an increased percentage of cavitated cells could be detrimental to the small intestine. The protective capacity of the mucus barrier could be altered after the stored mucins have been depleted. The rapid secretion of mucus is thought

Table 2. Bacterial Susceptibility to 5-FU

Organism	5-FU dose	Zone of inhibition (mm ± SEM)	*n*
Streptococcus pneumoniae	250 μg	Complete	
	100 μg	27.8 ± 0.09	1
	10 μg	21.8 ± 0.67	2
	1 μg	1.51 ± 0.37	3
	0.1 μg	Nil	
Staphylococcus aureus	250 μg	17.97 ± 0.38	3
	100 μg	15.70 ± 0.44	3
	10 μg	8.09 ± 0.29	3
	1 μg	Nil	
Pseudomonas aeruginosa	250 μg	Nil	
Escherichia coli	250 μg	Nil	
Staphylococcus epidermis	250 μg	27.18 ± 0.34	3
	100 μg	24.26 ± 0.90	3
	10 μg	16.29 ± 0.40	3
	1 μg	5.35 ± 0.08	3
	0.1 μg	Nil	
Enterococcus faecalis	250 μg	20.09 ± 0.36	3
	100 μg	17.68 ± 0.51	3
	10 μg	13.06 ± 0.76	3
	1 μg	3.47 ± 0.13	3
	0.1 μg	Nil	
Serratia marcescens	250 μg	7.40 ± 0.29	3
	100 μg	3.60 ± 0.17	3
	10 μg	Nil	
Bacteroides fragilis	250 μg	Complete	
	100 μg	Complete	
	10 μg	23.6 ± 1.51	3
	1 μg	Nil	
Clostridium botulinum	250 μg	19.33 ± 0.46	3
	100 μg	16.20 ± 1.29	3
	10 μg	13.99 ± 1.30	3
	1 μg	0.10 ± 1.10	3
	0.1 μg	Nil	
Lactobacillus acidophilus	250 μg	Complete	
	100 μg	Nil	
Bifidobacterium lactis	250 μg	Complete	
	100 μg	Complete	
	10 μg	5.11 ± 0.51	3
	1 μg	Nil	

to be caused by enteric neurotransmitters acting on epithelial cells (including goblet cells), such as acetyl choline (ACh), substance P (SP) and vasoactive intestinal peptide (VIP) (36), which suggests that 5-FU absorption by the intestine may cause an up-regulation of neurotransmitter release from enteric neurons, resulting in increased mucin secretion. Goblet cells and cavitated cells did not change significantly in the colon, suggesting no significant change to the protective capacity of mucins in the colon.

This study clearly demonstrated that changes in the microflora occur in the stomach, jejunum, colon and faeces. Culture methods showed decreases in *Clostridium spp*., *Escherichia spp*. and *Staphylococcus spp*. in the stomach, and decreases in the jejunum in *Clostridium spp*. and *Lactobacillus spp*., with an increase in *Escherichia spp*. 48–72 h after treatment. In the colon, *Clostridium spp*. and *Escherichia spp*. increased, but *Lactobacillus spp*. decreased. In the faeces, *Clostridium spp*. decreased, as did *Escherichia spp*. and *Proteus spp*. early, and *Streptococcus spp*. late. Being a qualitative technique, the culture method makes it difficult to gauge exactly how significant changes in bacteria are, to determine an increase in the amount of bacteria unless there was a small amount to begin with, and also to grow some fastidious organisms. Despite this, the technique gives a good indication of which organisms to target with other quantitative techniques. Real-time PCR using primers directed specifically at groups of bacteria in the intestine was performed on DNA from snap frozen faecal samples. This technique gave an accurate quantification of the amount of DNA for each group of organisms tested in the faeces of treated and control rats. *Clostridium spp*. and *Staphylococcus spp*. increased after treatment. Other changes seen included decreases in *Bacteroides spp*. and *Lactobacillus spp*., and a significant increase in *E. coli* ($P = 0.02$). It is likely that these changes are seen due to a direct effect on the bacteria in the GIT from 5-FU. Susceptibility tests for 5-FU on bacteria showed that *E. coli* and *P. aeruginosa* were not susceptible, even when applied at the manufacturer's concentration. All other bacteria tested were susceptible to varying degrees. 5-FU is absorbed to a degree in the GIT (4, 37), making it likely to have its effect on bacteria at this time. However, pharmacokinetic studies have shown concentrations of 5-FU given intraperitoneally to be lower in the peritoneal fluid and colon tissue than the concentrations the bacteria were susceptible to in this study (38), suggesting the implication of other mechanisms. Patients undergoing chemotherapy with 5-FU are susceptible to infections as the result of myelosuppression. Clinical data suggests that infections are often caused by bacteria found in the intestinal tract, such as *E. coli* and *P. aeruginosa* (39–42). *E. coli* and *P. aeruginosa* were shown not to be susceptible to 5-FU. Other organisms involved in maintaining a balanced microecology in the intestine were susceptible to 5-FU, which may allow the proliferation of non-susceptible bacteria and the opportunity for overgrowth and penetration of the damaged mucosa, causing host infections.

The loss of intestinal microflora also results in the loss of bacterial function in the gut, including processing of nutrients, regulation of intestinal angiogenesis, immune development in the GIT (11, 12), protection, and metabolism of enzymes, fatty acids, bile acids, cholesterol, steroid hormones and intestinal mucins (10). These are important functions in the GIT, and their lacking could also result in decreased GIT functional and protective capabilities. Probiotics are known to exert beneficial effects to the host when ingested, and therefore could be useful in controlling the intestinal microflora during chemotherapy (43). We have shown that VSL#3 is able to reduce the severity and duration of CID in rats treated with irinotecan (44). Further research into the role that probiotics could play in ameliorating mucositis should also be explored. The use

of probiotics could be a viable option for replacing and/or preventing lost microflora and maintaining gut homeostasis. The careful use of targeted antibiotics against *E. coli* and *Clostridium spp*. may also be useful.

The changes in electrolytes seen in this study are likely to be mucositis-related. The significant decrease in sodium at 48 h is likely to be due to gastrointestinal loss through decreased absorptive capacity as a result of damage caused by 5-FU. The significant increase in bicarbonate at 12 h is likely to be the result of a compensatory mechanism. 5-FU is broken down to urea, CO_2 and α-fluoro-β-alanine, all of which are excreted in urine (45). The temporary increase in blood CO_2 would be compensated by an excretion of H+ ions, and an increased reabsorption of HCO_3^-, thus resulting in an increased serum bicarbonate level (46).

In conclusion, 5-FU causes intestinal mucositis, the effects of which are predominantly seen in the small intestine. We have shown that 5-FU treatment results in changes to the intestinal microflora and mucin secretion, which may be responsible for the development of severe mucositis.

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