Rifaximin modulates the colonic microbiota of patients with Crohn’s disease: an in vitro approach using a continuous culture colonic model system

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Received 1 February 2010; returned 15 April 2010; revised 11 August 2010; accepted 13 August 2010

Objectives: Rifaximin, a rifamycin derivative, has been reported to induce clinical remission of active Crohn’s disease (CD), a chronic inflammatory bowel disorder. In order to understand how rifaximin affects the colonic microbiota and its metabolism, an in vitro human colonic model system was used in this study.

Methods: We investigated the impact of the administration of 1800 mg/day of rifaximin on the faecal microbiota of four patients affected by colonic active CD [Crohn’s disease activity index (CDAI ≥ 200)] using a continuous culture colonic model system. We studied the effect of rifaximin on the human gut microbiota using fluorescence in situ hybridization, quantitative PCR and PCR–denaturing gradient gel electrophoresis. Furthermore, we investigated the effect of the antibiotic on microbial metabolic profiles, using 1H-NMR and solid phase microextraction coupled with gas chromatography/mass spectrometry, and its potential genotoxicity and cytotoxicity, using Comet and growth curve assays.

Results: Rifaximin did not affect the overall composition of the gut microbiota, whereas it caused an increase in concentration of Bifidobacterium, Atopobium and Faecalibacterium prausnitzii. A shift in microbial metabolism was observed, as shown by increases in short-chain fatty acids, propanol, decanol, nonanone and aromatic organic compounds, and decreases in ethanol, methanol and glutamate. No genotoxicity or cytotoxicity was attributed to rifaximin, and conversely rifaximin was shown to have a chemopreventive role by protecting against hydrogen peroxide-induced DNA damage.

Conclusions: We demonstrated that rifaximin, while not altering the overall structure of the human colonic microbiota, increased bifidobacteria and led to variation of metabolic profiles associated with potential beneficial effects on the host.

Keywords: human gut model, inflammatory bowel disease, inflammatory bowel disorder

Introduction

Crohn’s disease (CD) is a chronic idiopathic inflammatory bowel disorder that is characterized by altered microbial composition, defective clearance of bacteria and enhanced mucosal uptake, resulting in increased immune stimulation. That the disease preferentially occurs in regions of the highest intestinal bacterial concentration indicates a role for commensal enteric bacteria in CD. While comprehensive culture- and molecular-based analyses of the microbiota of CD patients have failed to identify consistent enrichment of pathogenic species in CD tissues, recent studies have shown decreased microbial diversity of the intestinal ecosystem in CD patients, with a specific reduction in the concentration and diversity of members of the phyla Firmicutes and Bacteroidetes, which promote gastrointestinal health in multiple ways. Conversely, the abundance of Proteobacteria of the family Enterobacteriaceae was increased in the CD microbiota. It is not clear whether variation in these populations is a cause or a consequence of the disease.

Various therapeutic approaches have been employed to manage CD, including the administration of antibiotics. Metronidazole and ciprofloxacin are widely employed in clinical practice...
Rifaximin modulates the gut microbiota in an in vitro CD model

for treating perianal and colonic CD. However, the side effects of these antibiotics remain of concern, with long-term failure of treatment in over 20% of cases, leading to a continued search for effective antimicrobial agents. Rifaximin, a rifamycin derivative registered in many countries in Europe and the USA, has gained interest in the last decade for its pharmaco-toxicological and clinical characteristics. It has negligible intestinal absorption after oral administration, giving it an excellent safety profile. Its wide antimicrobial spectrum covers Gram-positive and -negative bacteria, including aerobes and anaerobes. Rifaximin has been successfully employed in the treatment of several intestinal disorders, including travellers’ diarrhoea, diverticular disease, and small intestine bacterial overgrowth. Recently, it has been reported that rifaximin, at the doses of 600 mg/day, 800 mg twice a day, and 1800 mg/day, induced clinical remission of active CD. These in vivo studies measured the efficacy of rifaximin in relation to the clinical score, but did not consider the impact of the antibiotic on the composition and metabolic activity of the human gut microbiota.

This study investigated the impact of the administration of 1800 mg/day of rifaximin on the faecal microbiota of four patients affected by colonic active CD [Crohn’s disease activity index (CDAI > 200)] using the continuous culture colonic model system. The three-stage continuous culture system is a useful tool to monitor the ecology and metabolic activities of the microbiota in the proximal, transverse and distal colon, in particular in relation to different environmental conditions, dietary intervention and the administration of drugs and antimicrobials. The influence of antibiotic treatment on the intestinal microbial ecology and bacterial metabolic profiles was determined and potential genotoxic and cytotoxic effects of rifaximin were assessed.

Materials and methods

Patients and drug

Four patients (Royal Berkshire Hospital, Reading, UK) affected by active CD (CDAI > 200) provided fresh faecal samples. None of the patients had received antibiotics or probiotics for at least 3 months before sampling, or steroids or other drugs with a proven impact on gut microbiota composition over the preceding 12 weeks. The antibiotic rifaximin (batch no. 07/071c) was kindly supplied by Alfa Wasserman SpA (Bologna, Italy).

Three-stage continuous culture colonic model system

The three-stage continuous culture model of the human colon (colonic model) consisted of three glass fermenters of increasing working volume, simulating the proximal (vessel 1 (V1), 280 mL), transverse (vessel 2 (V2), 300 mL) and distal (vessel 3 (V3), 320 mL) colon. V1 was fed by means of a peristaltic pump with complex colonic model growth medium (CMGM). The three fermenters were connected in series, with V1 feeding V2, which sequentially fed V3, finally overflowing to waste. All vessels were kept at 37°C, pH was controlled and kept at 5.5 (V1), 6.2 (V2) and 6.8 (V3), and the system was kept anaerobic by continuously sparging with O2-free N2. Foecal samples were collected on site, kept in an anaerobic cabinet (10% H2, 10% CO2, 80% N2) and used within a maximum of 15 min after collection. A 1:5 (w/w) dilution in anaerobic PBS (0.1 mol/L PBS (pH 7.4)) was prepared and the samples were homogenized in a stomacher (Seward, Worthing, UK) for 2 min. Each vessel was inoculated with 100 mL foecal slurry. Total system transit time was set at 36 h. After 24 h (T0) the medium flow was initiated and the system was run for eight full volume turnovers to allow steady state to be achieved (SS1). At SS1, samples were obtained on 3 consecutive days to confirm steady-state status by short-chain fatty acid (SCFA) and fluorescence in situ hybridization (FISH) analyses. Taking into account the operating volume (900 mL) and the retention time (36 h) of the colonic model system, 280 mg of rifaximin was added to V1 three times each day, equivalent to a human daily dose of 1800 mg antibiotic. Rifaximin was added to the system as described for a further eight volume turnovers, after which steady-state 2 (SS2) was achieved. Samples on 3 consecutive days were obtained to establish SS2 as described for SS1 (Figure S1, available as Supplementary data at JAC Online [http://jac.oxfordjournals.org/]). Samples for FISH were fixed immediately in 4% paraformaldehyde as previously described. Another sample set was centrifuged at 13000 g and the supernatant was stored at −20°C for cytotoxicity and genotoxicity analyses until the DNA was extracted. Samples for metabolomic analysis were frozen immediately.

Enumeration of bacterial populations by (FISH)

FISH was performed as described previously. The probes used are reported in Table 1 and were commercially synthesized and 5'-labelled with the fluorescent Cy3 dye (Sigma-Aldrich, St Louis, MO, USA).

Extraction of DNA from colon model system

Frozen samples recovered from the colonic model system were thawed and aliquots (250 μL) were centrifuged at 10000 g for 5 min, supernatants were removed and the samples washed in 1 mL PBS. After washing, the pellets were resuspended in 0.5 mL STE buffer (Tris 5 mmol/L pH 8.0; EDTA 0.5 mmol/L pH 8.0; NaCl 5 mmol/L). For each sample, lysozyme (4000 U) and mutanolysin (8 U) were added to the cell suspension and the sample was incubated at 37°C for 30 min. Protease K (6 U) and RNase (7 Kunitz units) were further added and samples incubated at 65°C for 1 h. After the addition of SDS (100 μL, 10% solution), samples were incubated for 15 min at 65°C and cooled on ice for ~30 min. One volume of phenol/chloroform/water (24:25:1; Applied Biosystems, Foster City, CA, USA) was added to the samples, which were mixed by inversion for 2 min and centrifuged for 10 min at 6000 g. The aqueous phase was transferred to a fresh tube, to which 1 mL of ice-cold ethanol was added. Samples were mixed by inversion, left on ice for at least 30 min and centrifuged at 10000 g for 5 min. The supernatants were carefully removed and DNA pellets were left to dry overnight. DNA was resuspended in 50 μL of sterile water, quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored at −20°C.

PCR–denaturing gradient gel electrophoresis (PCR-DGGE) and cluster analysis

Amplification of the V2–V3 region of the bacterial 16S rRNA gene, using the universal bacterial primers GCclamp-HDA1 and HDA2, and PCR-DGGE were performed as previously reported. Following electrophoresis, the gel was silver-stained and scanned using a Molecular Imager Gel Doc XR System (Bio-Rad). DGGE gel images were analysed using the FPOQuest Software Version 4.5 (Bio-Rad). In order to compensate for gel-to-gel differences and external distortion to electrophoresis, the DGGE patterns were aligned and normalized using an external reference ladder composed of known bacterial species. After normalization, bands were defined for each sample using the appropriate densitometric curves. Bands constituting less than 1% of the total band area were omitted from further analysis. Similarity between DGGE profiles was determined.
Table 1. Oligonucleotide probes used in this study for FISH analysis

<table>
<thead>
<tr>
<th>Target genus or group</th>
<th>Probe</th>
<th>Sequence (5' to 3')</th>
<th>Pre-treatment/% formamide</th>
<th>Hybridization/washing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Most bacteria</td>
<td>EUB338 I²</td>
<td>GCTGCTCCGCTGCTAGAGG</td>
<td>none</td>
<td>46–48</td>
</tr>
<tr>
<td>Most bacteria</td>
<td>EUB338 II²</td>
<td>GCAGCCACCCGCTAGGTT</td>
<td>none</td>
<td>46–48</td>
</tr>
<tr>
<td>Most bacteria</td>
<td>EUB338 III²</td>
<td>GCTGCCACCCGCTAGGTT</td>
<td>none</td>
<td>46–48</td>
</tr>
<tr>
<td>Atopobium cluster (Atopobium, Coriobacterium, Collinsella spp.)</td>
<td>Ato291</td>
<td>GTGCGCTCTCTCACC</td>
<td>none</td>
<td>50–50</td>
</tr>
<tr>
<td>Bacteroides spp.</td>
<td>Bac303</td>
<td>CAAATGGGGGGGACCTTT</td>
<td>none</td>
<td>46–48</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>Bif164</td>
<td>CATCCGGCATTACCACCC</td>
<td>none</td>
<td>50–50</td>
</tr>
<tr>
<td>Most Desulfovibrionales</td>
<td>DSV567</td>
<td>TACCGGATTCGCTCCT</td>
<td>15% formamide</td>
<td>46–48</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Enter1432</td>
<td>CTTTGGCACCACCT</td>
<td>none</td>
<td>46–48</td>
</tr>
<tr>
<td>Eubacterium rectale/Clostridium coccoides</td>
<td>Erec482</td>
<td>GCTTCTTAGTCGATGCCG</td>
<td>none</td>
<td>50–50</td>
</tr>
<tr>
<td>Faecalibacterium prausnitzii</td>
<td>Fprau64S</td>
<td>CCTCTGACACTCAAGAAAAC</td>
<td>45% formamide</td>
<td>46–48</td>
</tr>
<tr>
<td>Lactobacillus/Enterococcus spp.</td>
<td>Lab158</td>
<td>GTATTAGCAYCTGTCTTTCA</td>
<td>lysozyme</td>
<td>50–50</td>
</tr>
<tr>
<td>Clostridium sporopalaeoides</td>
<td>Rbo730²</td>
<td>TAAAGGCAGGCAGAGGC</td>
<td>lysozyme</td>
<td>55–55</td>
</tr>
<tr>
<td>bromii, Clostridium leptum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruminococcus albus, Ruminococcus flavifaciens</td>
<td>Rfla729²</td>
<td>AAAGCCGATGAAGCGCC</td>
<td>lysozyme</td>
<td>55–55</td>
</tr>
</tbody>
</table>

α,βThese probes were used together in equimolar concentrations.

by calculating the Pearson correlation. Clustering of the sample profiles was done using the unweighted pair-group method using arithmetic average (UPGMA).

Additionally, microbial ecology analysis was carried out by calculating the species richness and Shannon indices from the DGGE profiles. The species richness index is the observed number of species present in an ecosystem and represents the evenness of the species present in the ecosystem.24

Real-time quantitative PCR (qPCR)

qPCR was performed in a LightCycler instrument (Roche, Mannheim, Germany) and SYBR Green 1 fluorophore was used to correlate the amount of PCR product with the fluorescence signal. The Bifidobacterium genus-specific primer set Bif164/Bif66225 and the eubacterial primer set HDA1/HDA222 were used. Three sub-samples of each DNA extract were amplified as previously described.22,25 Bacterial and bifidobacterial DNAs were quantified using standard curves derived from known concentrations of genomic DNA from the sequenced strain Bifidobacterium longum NCC2705.26 Chromosomal DNA of the strains used as standards was extracted by using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) and serially diluted from 10⁵ to 10¹ molecules/μL. Results obtained by PCR were expressed as the percentage of bifidobacterial against eubacterial DNA.

Modulation of HT29 cell growth by rifaximin

The influence of colonic model supernatants before and after administration of rifaximin on the growth and survival of the human colon carcinoma cell line HT29 was determined using the growth curve assay.27

Briefly, HT29 cells, grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (w/v) fetal bovine serum and penicillin (50 IU/mL)/streptomycin (50 μg/mL), were harvested by trypsinization, adjusted to a concentration of 16x10⁶ cells/mL and seeded into 96-well plates yielding 8000 cells/well. After 24 h of incubation at 37°C in an atmosphere of 5% CO₂, sterile filtered colonic model supernatants and negative controls (PBS and CMGM) were added to the wells at concentrations of 0, 1, 2.5, and 10% (v/v). Following a further 72 h of incubation, cells were fixed and permeabilized with methanol for 5 min. The DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) for at least 30 min at 37°C in the dark, and the amount of DNA, which is proportional to the number of cells, was evaluated by fluorimetric analysis using a microplate reader (Tecan Systems, San Jose, CA, USA) with excitation and emission at 360 and 465 nm, respectively.

Results are expressed as EC₅₀, which represents the effective concentration of colonic model supernatants resulting in a 50% reduction of cell number under the specified cell culture and treatment conditions compared with the growth of untreated cells.

Determination of DNA damage by single-cell gel electrophoresis (Comet assay)

The influence of rifaximin on the DNA-damaging potential of the colonic model supernatants and negative controls (PBS and CMGM) were added to the wells at concentrations of 0, 1, 2.5, 5 and 10% (v/v). Following a further 72 h of incubation, cells were fixed and permeabilized with methanol for 5 min. The DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) for at least 30 min at 37°C in the dark, and the amount of DNA, which is proportional to the number of cells, was evaluated by fluorimetric analysis using a microplate reader (Tecan Systems, San Jose, CA, USA) with excitation and emission at 360 and 465 nm, respectively.

Results are expressed as EC₅₀, which represents the effective concentration of colonic model supernatants resulting in a 50% reduction of cell number under the specified cell culture and treatment conditions compared with the growth of untreated cells.

Samples were centrifuged at 280 g for 5 min at 4°C and washed once with PBS. Three 50 μL aliquots of each sample were centrifuged again and the resulting cell pellets were re-suspended in warm, low melting point agarose and further processed as described.29 Numbers and viability of HT29 cells were assessed before and after incubation using the Trypan Blue exclusion assay.
Gas chromatography–mass spectrometry/solid-phase microextraction analysis (GC-MS/SPME)

GC-MS/SPME was performed as previously described.30 GC-MS analyses were performed on an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 5975C mass selective detector operating in electron impact mode (ionization voltage 70 eV). A Supelcowax 10 capillary column (60 m length, 0.32 mm inner diameter) was used (Supelco). Identification of the molecules detected in culture broths was based on a comparison of their retention times with those of pure compounds (Sigma-Aldrich) analysed in the same conditions. The identification was further confirmed by comparing mass spectra of compounds with those contained in available databases (National Institute of Standard and Technology version 2005 and Wiley version 1996) and in the literature,31 as well as with those of pure standards. Quantitative data of the identified compounds were obtained by interpolation of the relative areas versus the internal standard area, in calibration curves built with pure reference compounds. The concentration of volatile compounds for which there were no pure references was obtained by using the calibration graphs of the compounds with the most similar chemical structure.

1H-NMR spectroscopy

The water-soluble fraction of culture broth was studied by 1H-NMR spectroscopy.32 1H-NMR spectra were acquired on the collected supernatants, with no further treatments, at 300 K on a Mercury-plus NMR spectrometer (Varian, Palo Alto, CA, USA), operating at a proton frequency of 400 MHz. Residual water signal was suppressed by means of presaturation.

1H-NMR spectra were processed by means of VNMRJ 6.1 software from Varian.

Statistical analysis

All data were analysed by one-way ANOVA, using Tukey post-test analysis when the overall P value of the experiment was below the value of

Figure 1. Bacterial groups detected by FISH in the culture broths recovered from the three different vessels (V1, V2 and V3) of the colonic model system before (SS1) and after (SS2) rifaximin treatment. Results are reported as means of the data of four colonic models ± SEM. For each colonic model, measurements were performed in triplicate (days 14, 15 and 16). **P<0.01; ***P<0.001.
significance ($P<0.05$). An additional paired t-test was applied in order to assess the significance of results of single pairs of data. Analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

**Results**

**Impact of rifaximin on the gut microbiota composition**

Figure 1 shows the FISH counts of the 12 bacterial groups reported in Table 1 for each vessel at SS1 and SS2. Total bacteria counts (EUB338 1-I-III) remained stable after the antibiotic treatment (SS2), showing that rifaximin did not modify the overall gut bacterial concentration. *Eubacterium rectale/Clostridium cocooides* (Erec482) was the predominant group at SS1, while *Bacteroides* (Bac303) was the second most represented genus. Notably, levels of both Erec482 and Bac303, as well as *Enterobacteriaceae*, lactobacilli, *Ruminococcus* spp. and sulphate-reducing bacteria, did not show any significant modification after rifaximin treatment (SS2). A different behaviour was determined for *Bifidobacterium*, *Atopobium* cluster and *Faecalibacterium prausnitzii* (Figure 1). FISH analysis demonstrated that *Bifidobacterium*, a predominant health-promoting genus of the human gut microbiota, increased significantly after rifaximin treatment in all three vessels (2.5- to 3-fold). *Atopobium* cluster increased significantly in V2 and in V3 (1.4- to 1.6-fold), while no significant modification occurred in V1. *F. prausnitzii*, a suggested probiotic group with anti-inflammatory properties, increased significantly in concentration in V3 (1.4-fold).

The increase in *Bifidobacterium* spp. due to the antibiotic treatment was further confirmed using qPCR with genus-specific primers (Figure 2). The similarity of the bifidobacterial concentrations determined by qPCR and FISH ($P>0.05$) emphasizes the reliability of the results.

PCR-DGGE analysis showed that the biodiversity of the gut microbiota was not influenced by rifaximin administration, as assessed by the richness and Shannon indices ($P>0.05$). Mean values of the richness index for the specific colonic model vessels were: V1 (SS1: 17 versus SS2: 19.3), V2 (SS1: 19 versus SS2: 19.8) and V3 (SS1: 17.3 versus SS2: 17.8). Mean values of the Shannon index were: V1 (SS1: 2.65 versus SS2: 2.69), V2 (SS1: 2.62 versus SS2: 2.71) and V3 (SS1: 2.63 versus SS2: 2.56). Furthermore, the peak heights of DGGE densitometric curves were analysed using the Mann–Whitney U-test, in order to test if single-species abundances were affected by the anti-biotic treatment. No significant changes in species abundance were found when comparing SS1 and SS2.

Cluster analysis of DGGE profiles confirmed the stability of the human gut microbiota structure during the antibiotic treatment period (Figure 3). Four distinct groupings were obtained, corresponding to the DGGE profiles of the four CD patients. Analysis of similarity using the Pearson coefficient revealed a high inter-individual diversity. The similarity index (SI) ranged from 20.8% to 43.4% in SS1 (mean value $34.7 \pm 3.9$) and from 18.5% to 45.5% in SS2 (mean value $32.2 \pm 4.6$).

*Bifidobacterium*-specific PCR-DGGE analysis showed that *B. bifidum* and *B. breve* were the predominant species in all samples recovered from the colonic model systems at SS1 and SS2 (data not shown).

**Genotoxic effects of colonic model supernatants**

No significant differences were observed in Comet tail intensity between SS1, SS2 and PBS (as a non-genotoxic agent), indicating that rifaximin did not exert a genotoxic effect (Figure 4a). Co-incubation of colonic model supernatants and *H. pylori* a potent genotoxic agent, resulted in a significantly reduced tail intensity for samples taken after the administration of rifaximin in V2 compared with SS1 (Figure 4b). These in vitro results indicate an antigenotoxic effect of the fermentation supernatants in the presence of rifaximin, suggesting an in vivo chemoprotective function towards epithelial cells.

**Cytotoxic effects of colonic model supernatants**

EC$_{50}$ was used to compare the effects of supernatants on cell growth (Figure 5). No significant changes between EC$_{50}$(SS1) and EC$_{50}$(SS2) were found for V1 and V3. Conversely, V2 colonic model supernatants after the administration of rifaximin were significantly less cytotoxic than before (EC$_{50}$, SS1: 3.35 versus SS2: 4.50; $P=0.022$).

**Metabolic profiles of colonic models**

Metabolites identified by $^1$H-NMR and GC-MS/SPME, whose concentrations were significantly influenced by rifaximin, are reported in Tables 2 and 3, respectively. These metabolites belong to the chemical classes of SCFAs and their derivatives, alcohols, amino acids, ketones and aromatic organic compounds. Concentrations of SCFAs increased, as did those of propanol, decanol, nonanone and aromatic organic compounds, whereas ethanol, methanol and glutamate decreased.
Discussion

Increasing evidence suggests that the dynamic balance between the bacterial species of the human intestinal microbiota and host defence plays a pivotal role in the initiation and pathogenesis of chronic CD. Furthermore, metabolomic studies have indicated that the disruption of the normal bacterial ecology in CD induces pronounced modifications in the pattern of metabolites synthesized by microbiome activity.

Clinical studies have demonstrated the efficacy of rifaximin in inducing remission in patients affected by CD, even if the impact of this antibiotic on the human microbiome is not well established.

The feasibility of the three-stage continuous fermentative colonic model system assessing the impact of antibiotics on the intestinal microbiota has been demonstrated recently for fluoroquinolones, vancomycin, clindamycin, oritavancin, ureidopenicillin/β-lactamase inhibitor and cefotaxime and its derivative.

DGGE analysis, which provides a semi-qualitative fingerprint of the faecal ecosystem, showed a complex but overall relatively stable and unique DGGE profile for each patient, with high SI values between SS1 and SS2 profiles, and no clustering of band patterns according to the rifaximin treatment. FISH analysis demonstrated that rifaximin fails to alter the concentration of
the principal bacterial groups constituting the core gut microbiota, but increases were found for *Bifidobacterium*, *Atopobium* cluster and *F. prausnitzii*.

*Bifidobacteria*, widely used as probiotics, have been suggested to play a beneficial role in the management of clinical inflammatory bowel disease due to their effects on epithelial cell function and intestinal health, including enhanced epithelial barrier function, modulation of epithelial cytokine secretion into an anti-inflammatory dominant profile, altered mucus production, modification of the innate and systemic immune systems, and induction of regulatory T cell effects.

Recently, it has been demonstrated that levels of *F. prausnitzii*, a major member of the *Clostridium leptum* group and one of the most prevalent bacteria within the human gut, decreases significantly in patients affected by CD. Therefore, it has been suggested that *F. prausnitzii* could have a role in gut homeostasis, taking into account its immunomodulatory activities. The *Atopobium* cluster comprises bacteria of the Coriobacteriaceae family, belonging to the genera *Coriobacterium*, *Atopobium* and *Collinsella*. They form an interesting group of numerically important bacteria in the human intestinal tract.

The in vitro rifaximin susceptibility of several bacterial groups composing the human gut microbiota indicates that some intestinal symbionts can develop rifaximin resistance following exposure to rifaximin. Indeed, members of the most relevant microbial groups analysed in the present study, such as *Bacteroides* spp., *Lactobacillus* spp., *Clostridium/Eubacterium* spp., *Bifidobacterium* spp. and *Atopobium/Collinsella* spp., were able to grow at rifaximin concentration values higher than 1024 mg/L.

These results are in agreement with those obtained in a previous clinical study performed by administering the same dose of rifaximin (1800 mg/day) to patients affected by ulcerative colitis. Culture-dependent analysis of faecal samples showed that the antibiotic did not alter the concentration of some major bacterial groups, while it induced an increase in

**Figure 5.** Cytotoxic effect of supernatants recovered from V1 (a), V2 (b) and V3 (c) of the colonic model system before (SS1) and after (SS2) rifaximin treatment. Cytotoxicity was assessed by co-incubating HT29 cells with increasing concentrations (0%, 1%, 2.5%, 5% and 10%) of fermentation supernatants followed by DAPI staining. Results are expressed as means of relative HT29 cell growth (%) of four colonic models ±SEM. For each colonic model, measurements were performed in triplicate. EC50 values were calculated from the growth curves shown in (a), (b) and (c) for SS1 and SS2; (d) shows the comparison of EC50 values in SS1 and SS2 for each vessel. *P*<0.05.
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**Table 2.** Assessment by $^1$H-NMR of metabolites whose concentrations changed significantly after rifaximin treatment

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>$^1$H (ppm)</th>
<th>$^1$H groups</th>
<th>Before treatment (SS1)</th>
<th>After treatment (SS2)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>4.1</td>
<td>H2</td>
<td>0.052 ± 0.007</td>
<td>0.089 ± 0.014</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.9</td>
<td></td>
<td>14.5 ± 0.7</td>
<td>15.9 ± 0.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Propionate</td>
<td>1.09</td>
<td>H3</td>
<td>4.0 ± 0.2</td>
<td>4.2 ± 0.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.61</td>
<td>H1</td>
<td>2.1 ± 0.4</td>
<td>1.6 ± 0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Methanol</td>
<td>3.38</td>
<td></td>
<td>1.11 ± 0.07</td>
<td>0.99 ± 0.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.61</td>
<td>H2</td>
<td>3.8 ± 0.9</td>
<td>2.9 ± 0.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.76</td>
<td></td>
<td>4.9 ± 0.4</td>
<td>3.8 ± 0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Phenyalanine</td>
<td>7.42</td>
<td>H2</td>
<td>0.17 ± 0.28</td>
<td>0.16 ± 0.25</td>
<td>0.396</td>
</tr>
<tr>
<td>Methanol</td>
<td>3.29</td>
<td>H2</td>
<td>0.15 ± 0.04</td>
<td>0.14 ± 0.04</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Analysis was performed on a pool of culture broths recovered from V1, V2 and V3 of the colonic model system. Data are presented as area-normalized intensities of representative metabolite signals. Results are reported as means of the data related to four colonic models ± SEM.

**Table 3.** Assessment by GC-MS/SPME of metabolites whose concentrations changed significantly after rifaximin treatment

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Before treatment (SS1)</th>
<th>After treatment (SS2)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propanol</td>
<td>2.25 ± 0.27</td>
<td>3.93 ± 0.51</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Decanol</td>
<td>0.05 ± 0.01</td>
<td>0.18 ± 0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Phenyl ethanol</td>
<td>1.49 ± 0.31</td>
<td>2.92 ± 0.53</td>
<td>0.01</td>
</tr>
<tr>
<td>Nonanone</td>
<td>0.14 ± 0.02</td>
<td>0.37 ± 0.06</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Indole</td>
<td>42.90 ± 6.28</td>
<td>70.53 ± 9.64</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Methyl phenylacetate</td>
<td>0.19 ± 0.03</td>
<td>0.41 ± 0.03</td>
<td>0.001</td>
</tr>
<tr>
<td>3-Methyl thiphene</td>
<td>0.05 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>3-Thiophene acetic acid</td>
<td>1.05 ± 0.02</td>
<td>2.09 ± 0.24</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Analysis was performed on a pool of culture broths recovered from V1, V2 and V3 of the colonic model system. Data are presented as the ratio between the relative peak area of the metabolite of interest and the relative peak area of an internal standard at a defined concentration. Results are reported as means of the data related to four colonic models ± SEM.

Bifidobacteria. A recent study on the use of rifaximin (800 mg/day) in the treatment of travellers’ diarrhoea further confirmed that the clinical effectiveness of the antibiotic is not associated with evident alterations of the colonic microbiota.48

Alternative mechanisms of action that do not involve a direct bactericidal activity have been suggested to explain the efficacy of rifaximin: (i) alteration of virulence factors of enteric bacteria;48 (ii) reduction of pathogen adhesion and internalization to intestinal epithelium;49 and (iii) reduction of inflammatory cytokine release50–51.

Combining the results obtained by GC/MS-SPME and $^1$H-NMR, increases in SCFAs (acetate and propionate) and lactic acid were detected after rifaximin treatment. SCFAs play an important role in intestinal health, providing energy to the intestinal mucosa and promoting epithelial cell growth.52 Bifidobacteria are reported to metabolize dietary oligosaccharides into SCFAs, especially acetate.53 Propionate is produced mainly by Bacteroidetes, but also by some members of Firmicutes (families Clostridiaceae, Lactobacillaceae and Streptococcaceae), while lactate is a main fermentation product of the families Bifidobacteriaceae and Coriobacteriaceae.54 These modifications of metabolic profiles may be related to the increase in concentration of bifidobacteria, Atopobium cluster and F. prausnitzii demonstrated during treatment.

Proteins and amino acids are fermented by the gut ecosystem to a variety of important metabolites, including mostly...
branch-chain fatty acids (BCFAs), ammonia, hydrogen sulphides, polyamines and indolic and phenolic compounds. Protein metabolites produced by the microbiota are mostly known for having deleterious effects on colonic physiology. However, direct proof of such effects in humans is lacking. After rifaximin treatment, we demonstrated an increase in the concentration of tyrosine and decreases in glutamate and phenylalanine. An increase in tyrosine was also reported in a mouse model inoculated with human baby microbiota, in which an increase in Bifidobacterium was shown following the administration of probiotics and synbiotics. Furthermore, we showed that nonanone and indole, metabolites derived from protein metabolism, were increased during antibiotic administration. These compounds have been demonstrated to be depleted in individuals with Clostridium difficile and Campylobacter jejuni infection, and in the presence of gastrointestinal pathologies such as ulcerative colitis.

Whilst metabolites derived by fermentative metabolism of carbohydrates and proteins are well studied, little is known about the role of alcohols. Our results demonstrate that concentrations of propanol and decanol increased whereas ethanol and methanol decreased during antibiotic treatment. These results are promising in the light of the findings of Garner and co-workers, which demonstrated that propanol concentration decreased in subjects with C. difficile infection and ulcerative colitis, decanol was absent in pathological conditions and methanol, increased especially in subjects affected by C. jejuni infection and ulcerative colitis. Our findings enforce the hypothesis formulated by Jiang et al. that rifaximin could act by means of non-conventional mechanisms, probably involving regulation of the metabolic activities of the gut microbial communities.

In the present work, we evaluated the potential genotoxic and cytotoxic effects of rifaximin towards HT29 human epithelial colonic cancer cells. Our results show that rifaximin does not induce cytotoxicity. Furthermore, it does not exert any genotoxic action itself. In contrast, colonic model supernatants from V2 and V3 showed a pronounced anti-genotoxic effect against hydrogen peroxide. This effect could be due to the increase in bifidobacteria as it has been shown that cell-free fermentation supernatants of bifidobacteria protect colon cells from DNA damage induced by N-methyl-N'-nitro-N-nitrosoguanidine and faecal water. However, a direct scavenging effect of rifaximin could also be a possible mechanism of exerting protection against hydrogen peroxide-induced DNA damage.

In conclusion, the employment of a continuous culture colonic model system allowed us to broadly investigate the impact of rifaximin on the colonic microbiota of CD patients. We demonstrated that rifaximin at the concentration tested neither disrupts the overall biostructure of the human microbiota nor exerts any cytotoxic or genotoxic activities, but it does provoke changes in bacterial metabolism and bifidobacterial numbers that support a functional advantage to the host.

Transparency declarations
F. C. is a member of the Pharma-toxicological Development Department of Alfa Wassermann Spa, which supplied rifaximin. All other authors: none to declare.

Supplementary data
Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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Funding
This study was supported by a grant from the Italian Ministry of Education, University and Research (MIUR).
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