The Intestinal Microbiota and Gut Health:

Contribution of the Diet, Bacterial Metabolites, Host Interactions and Impact on Health and Disease

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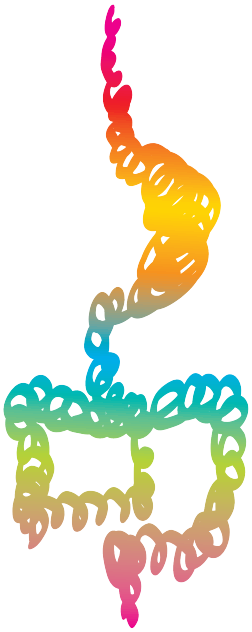

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Measuring in vitro, the ability of a prebiotic and polyphenol combination to beneficially modulate the human gut microbiota and protect against unwanted antibiotic side effects. A. Koutsos, L. Conterno, M. Lima, F. Fava, R. Viola, and K. Tuohy, *Department of Food Quality and Nutrition, Fondazione Edmund Mach, IASMA Research and Innovation Centre, Via E. Mach 1, 38010 S. Michele (TN), Italy*

Introduction

The gut microbiota, comprising many thousands of microorganisms, constitutes an important barrier to ingested pathogens, affects the development and function of the immune system and contributes to the co-metabolic processes with the host, most notably the enterohepatic circulation of bile acids and co-metabolism of complex plant polysaccharides and polyphenols. However, antibiotics can disrupt these beneficial activities. Although they remain our first line therapy against bacterial pathogens and an essential weapon in modern medicines fight against infectious disease, antibiotics can have side effects (1). A proportion of orally ingested antibiotics may escape absorption in the stomach and upper gut, and inadvertently reach non-target intestinal sites such as the colon, home to the vast majority of the human gut microbiota. Diarrhoea is a common side effect of many oral antibiotic therapies and in its most extreme, can lead to *Clostridium difficile* associated diarrhoea (CDAD). *Clostridium difficile* is a spore-forming biofilm generating bacterial pathogen naturally more resistant to antibiotics than other gut bacteria is now recognised as a "super-pathogen" responsible for many nosocomial infections in modern hospitals. In this small in vitro experiment, we investigated the ability of different functional food approaches to first of all to fortify the human gut microbiota and secondly, to protect the gut microbiota from the unwanted side effects of residual broad spectrum antibiotic.

Material and Methods

Anaerobic batch cultures (pH 6.8, 37 ºC) were used to evaluate the microbiota modulatory abilities of abilities of different treatment regimes: (i) prebiotic (inulin 1% w/v), (ii) a herbal mix (0.1 % w/v, a mixture of propolis, olive and thyme extracts provided by Aboca Srl. Italy), (iii) inulin plus the herbal mix (prebiotic + herbal mix, 15 and 0.1% respectively), (iv) prebiotic plus antibiotic, and (viii) prebiotic plus herbal mix and antibiotic and (ix) untreated faecal inoculum (control). Antibiotic, augmentin (amoxicillin and clavulanic acid) was always added at 10% w/v per 200 ml reaction vessel of the commonly used daily dose, 250mg/125mg. *In-vitro* fermentations were conducted in triplicate using faecal samples collected from three healthy, male subjects aged between 30 and 47, as described in Connolly *et al.* (2012) (2). A 5 ml sample from each vessel was taken immediately for analysis similarly samples were taken at 5, 10, 24 and 48 hours.

The enumeration of microbial populations was carried out using fluorescently labelled 16S rRNA targeted oligo-nucleotide probes and fluorescent *in situ* hybridisation (FISH). Oligonucleotide probes; Bif164, specific for the *Bifidobacterium* genus; Lab158, for the *Lactobacillus*– Enterococcus group; Bac303, specific for the *Bacteroides* and *Prevotella* group; His150, for the *Clostridium histolyticum* subgroup; Erec482 for the *Ruminococcus–Eubacterium–Clostridium* (EREC) cluster; FPrau for *Faecalibacterium prausnitzii*, Ent for

enterobacteria and Cdif *Clostridium difficile*, were chosen based on the high abundance of these bacteria as part of the whole bacterial populations of the colonic microbiota.

Results and Discussion

Total bacteria varied little over the course of the experiment regardless of treatment, prebiotic, antibiotic, herbal mix or mixed treatments. Numbers of bifidobacteria increased significantly over time up to 10h for the inulin fermentation and a similar level was observed after 24h in the inulin plus herbal mix fermentation. This may indicate that the herbal mix, while achieving the same level of bifidogenesis in the presence of inulin, did so at a slower rate, a trait which if translated to the human situation, might indicate extension of beneficial prebiotic activities into the distal colon where it may have added health promoting effects (3). In all cases, the presence of the antibiotic inhibited bifidogenesis and indeed reduced bifidobacterial numbers. This was particularly apparent in the inulin plus antibiotic fermentation, which at time 48h, gave a lower level than the herbal mix, inulin plus antibiotic.

For *Bacteroides* spp., a statistically significant reduction in numbers was observed after 24h for the herbal mix, inulin and antibiotic fermentation. A similar reduction was observed in inulin plus antibiotic, but this was not statistically significant probably because of slightly lower starting levels of *Bacteroides*. Other fermentations showed reductions in *Bacteroides* populations at 24 or 48 hours, while small increases were found in the inulin, inulin plus antibiotic and herbal mix fermentation vessels at 10 h. Comparing the herbal mix with inulin in the presence or absence of antibiotic, the herbal mix plus inulin gave slightly lower numbers of *Bacteroides* through out the incubation period, which in the presence of the antibiotic, the herbal mix plus inulin appeared to protect *Bacteroides* numbers up until 10 h. However, these small changes did not appear to be statistically significant. *Bacteroides*, a dominant Gram negative genus within the human colon, includes both pathogenic and commensal species. Ley *et al.* (2006) (4) were the first to show that obese humans have a reduced Bacteroidetes to Firmicutes ratio. These data do not directly relate to numbers of *Bacteroides* enumerated by the FISH probe we have used here as they also include the Prevotella. De Filippo *et al.* (2010) (5) more recently, showed that people following a Western-style diet had much reduced relative abundance of Prevotella compared to populations following traditional, ancestral diets in Africa, rich in plant foods, fiber and polyphenols and low in fat, refined sugars and meat.

No change or a small reduction in numbers of *F. prausnitzii* was observed in the inulin, faeces only, inulin plus antibiotic, and herbal mix with inulin plus antibiotic. A small, though not statistically significant, increase in *F. prausnitzii* was observed in the herbal mix plus inulin vessel, indicating that the herbal mix may have an added stimulatory effect on this important species within the gut microbiota in the presence of fermentable substrate. *F. prausnitzii* is an important butyrate producing bacterium, which has been suggested to play a protective role in inflammatory bowel disease (6).

Statistically significant differences were observed in numbers of enterobacteria between the different fermentations after 48h of incubation. Generally, numbers did not change greatly for the negative control (faeces only) and inulin fermentations. The antibiotic treatment reduced numbers of enterobacteria regardless of treatment. Interestingly, the herbal mix plus inulin appeared to have a greater inhibitory effect against the enterobacteria than inulin alone.

Bacterial populations (log_{10} cells/ml batch culture fluid) in pH-controlled and stirred batch cultures at 0, 5, 10, 24 and 48 h at different treatments.

		Bif		F.Prau		Bac		Enter		Lab		Erec		C.His		C.Dif		Total	
Treatment	Time (h)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Only faeces	0	7.8	0.3	8.2	0.1	8.2	0.3	7.4	0.7	8.0	0.3	8.4	0.3	8.6	0.3	6.9	0.1	9.0	0.2
	5	8.0	0.8	8.1	0.2	8.3	0.3	7.5	0.4	7.9	0.0	8.2	0.3	8.6	0.3	6.9^{ab}	0.1	8.8	0.2
	10	7.8	0.2	8.0	0.1	8.3	0.5	7.3	0.1	8.0	0.1	8.2	0.1	8.5	0.2	6.9	0.4	8.8	0.2
	24	7.8	0.3	7.8	0.3	7.8	0.0	7.5	0.2	7.6	0.4	8.1^{abc}	0.2	8.6	0.1	6.5	0.8	8.8	0.1
	48	7.7	0.3	7.9	0.4	7.5	0.2	7.3^{abc}	0.2	7.7	0.4	8.2	0.2	8.5	0.2	6.3	0.5	8.8	0.4
Inulin	0	7.9	0.2	8.2	0.1	7.8	0.2	7.3	0.7	8.1	0.4	8.3	0.2	8.6	0.2	6.9	0.1	9.1	0.2
	5	8.8	0.4	8.1	0.3	7.7	0.2	7.4	0.2	7.9	0.1	8.3	0.2	8.6	0.3	6.9^{ab}	0.2	8.8	0.3
	10	9.0*	0.4	8.1	0.3	8.1	0.1	7.2	0.8	8.0	0.3	8.4	0.3	8.8	0.6	7.0	0.6	9.0	0.3
	24	8.8	0.2	8.2	0.3	8.0	0.4	7.2	0.6	7.8	0.2	8.5^{c}	0.3	8.7	0.6	7.3	0.9	9.0	0.2
	48	8.5	0.6	8.2	0.4	7.9	0.4	7.5^{a}	0.3	7.9	0.0	8.4	0.5	8.6	0.5	6.8	0.9	9.0	0.2
Inulin, antibiotic	0	7.8	0.5	8.2	0.2	7.8	0.3	7.3	0.5	8.1	0.2	8.2	0.2	8.8	0.3	6.8	0.0	8.9	0.1
	5	7.9	0.6	8.0	0.3	7.4	0.6	7.3	0.9	7.9	0.2	8.1	0.4	8.6	0.4	6.7^{ab}	0.1	8.8	0.2
	10	7.8	0.8	7.9	0.2	8.3	0.5	7.0	0.5	8.0	0.1	8.2	0.3	8.5	0.4	6.9	0.2	8.9	0.2
	24	7.6	0.4	7.7	0.2	7.5	0.7	6.3	0.5	7.7*	0.1	7.8^{ab}	0.3	8.2	0.4	6.6	0.4	8.6	0.2
	48	7.3	0.8	7.5	0.4	7.3	0.4	6.6^{bc}	0.1	7.9	0.2	8.0	0.3	8.1	0.4	6.5	0.4	8.4	0.3
Herbal mix, inulin	0	7.9	0.5	8.2	0.1	7.8	0.3	7.4	0.7	8.0	0.2	8.2	0.1	8.9	0.2	6.9	0.1	9.0	0.2
	5	8.1	0.8	7.9	0.3	7.4	0.3	7.4	0.6	7.9	0.0	8.2	0.3	8.6	0.3	6.4^{abc}	0.1	8.7	0.3
	10	8.6	0.7	8.2	0.4	8.0	0.1	7.2	0.9	8.0	0.2	8.4	0.4	8.7	0.5	6.8	0.6	9.0	0.4
	24	8.9	0.5	8.5	0.3	7.7	0.1	6.7	0.4	7.7	0.2	8.4^{bc}	0.2	8.5	0.1	6.5	0.2	8.8	0.4
	48	8.0	1.0	8.3	0.2	7.6	0.4	6.9^{abc}	0.5	7.6	0.1	8.3	0.5	8.4	0.3	6.3	0.3	9.0	0.4
Herbal mix, inulin, antibiotic	0	8.2	0.1	8.1	0.0	8.1	0.3	7.7	0.2	8.0	0.2	8.3	0.2	8.8	0.3	6.7	0.4	8.5	0.2
	5	8.6	0.7	7.9	0.3	8.2	0.3	7.7	0.3	8.0	0.1	8.2	0.3	8.5	0.3	6.1^{c}	0.3	8.4	0.1
	10	7.7	0.8	7.9	0.3	8.3	0.2	6.7	1.2	8.1	0.1	8.1	0.2	8.7	0.3	6.6	0.4	8.9	0.3
	24	7.8	0.9	7.9	0.5	7.4*	0.3	6.7	0.6	7.6	0.2	8.2^{abc}	0.2	8.4	0.3	6.6	0.5	8.5	0.1
	48	7.5	0.5	7.6	0.5	7.4*	0.4	7.0^{abc}	0.4	7.7	0.2	7.9	0.2	8.4	0.4	6.1	0.4	8.6	0.2

Univariate analysis of variance was used to analyse the effects of Treatment and Time on the bacterial population. When statistical differences were found, data were further tested by the Tukey post hoc test. Mean value (*) was significantly different from that at 0 h: $P<0.05$. Mean value for the same Time point with unlike superscript letters was significantly different between the different treatments: $P<0.05$

Neither the antibiotic nor the functional food treatments appeared to have any impact on numbers of *Lactobacillus/Enterococcus* over the course of the fermentation study. A small decrease in numbers was observed in all fermentations after 10 hours, but this change was not significant compared to starting levels, apart from the fermenter dosed with inulin plus antibiotic, where the drop in lactobacilli/enterococci numbers was statistically significant at 24 h.

Small but not stastistically significant changes in numbers of *Eubacterium rectale* were observed in most vessels over the course of the experiment. Inulin and the herbal mix plus inulin tended to increase numbers up to 24h, while the antibiotic tended to give reduced numbers at 48h. There was a dip in numbers at 24h for the inulin plus antibiotic vessel which did not appear until 48h for the herbal mix plus inulin and antibiotic vessel.

Numbers of *Clostridium histolyticum/perfringens* group remained fairly stable over the course of the experiment and did not appear to be effected by either the functional foods nor presence of the antibiotic. Numbers of *Clostridium difficile* decreased in all vessels over the course of the experiment. Small, statistically significant differences between treatments after 5 hours were not evident at later time points, indicating that these effects were transient and may not necessarily be representative.

In conclusion, the herbal mixture plus inulin significantly modulated the composition and activity of the human gut microbiota in a manner similar to inulin alone, but with some additional herbal mix features, which could indicate enhanced health effects. The mix plus inulin also induced small, though favourable changes within key bacterial groups in a slightly different manner than inulin, specifically slowing bifidogenesis induced by inulin, which in humans might extend prebiotic activity into the distal colon, slightly higher levels of the butyrate producing, anti-inflammatory microorganism *Faecalibacterium pruasnitzii* compared to inulin alone and apparent protection of *Eubacterium rectale* group against antibiotic compared to the inulin alone. There was also some suggestion from the data of an apparent increased anti-enterobacterial activity of the herbal mix plus inulin compared to inulin alone. However, many of these differences within the gut microbiota was of a similar magnitude as those seen within inulin and further investigations, particularly in animals or in

human subjects are required to confirm the beneficial modulation of the gut microbiota observed for the herbal mix plus inulin.

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