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Effects of probiotics on the composition of the intestinal microbiota following antibiotic therapy

Susan F. Plummer[a,*], Iveta Garaiova[a], Tinnu Sarvotham[b], Simon L. Cottrell[c], Stephanie Le Scouiller[d], Mark A. Weaver[a], James Tang[a], Philippa Dee[e], John Hunter[e]

[a] *Cultech Biospeciality Products, Research Department, York Chambers, York Street, Swansea SA1 3NJ, UK*
[b] *University of Wales, College of Medicine, Cardiff CF14 4XN, UK*
[c] *Cardiff School of Biosciences, Cardiff University, Cardiff CF10 3XQ, UK*
[d] *School of Construction Management and Engineering, The University of Reading, Reading RG6 6AH, UK*
[e] *Addenbrooke's Hospital, Cambridge CB2 2QQ, UK*

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Abstract

The effects of probiotic supplementation on the intestinal re-growth microbiota following antibiotic therapy were studied in a double-blind placebo-controlled study. In the placebo group, numbers of facultative anaerobes and enterobacteria increased significantly, and at day 35 the numbers were significantly higher in the placebo group than in the active group; in the active group, the numbers of bacteroides increased significantly. Although the numbers of enterococci in both groups did not change, in the placebo group the number of patients harbouring antibiotic-resistant enterococci post therapy increased significantly. There was no change in the incidence rate of antibiotic resistance among the patients in the probiotic group.

Keywords: Intestinal microbiota; Antibiotics; Probiotics; Antibiotic resistance

1. Introduction

The bacterial flora of the gastrointestinal tract play a major role in human physiology, modulating metabolic and immunological processes and providing colonisation resistance, which is the prevention of overgrowth of opportunistic microorganisms. Administration of antimicrobial agents, whether therapeutically or prophylactically, disturbs the ecological balance between the host and the normal microbiota [1]. The extent of the disturbance depends on the nature of the antimicrobial agent, the absorption, the route of elimination and any potential enzymatic degradation and/or binding to faecal material. However, predicting the effects of an antibiotic on the microbiota can be difficult due to the complex relationships among the components of the microbiota [2].

Disturbance of the microbiota is frequently associated with diarrhoea, gastritis, glossitis and pruritus [3] as well as fungal infections. In addition, altered sensitivity to secondary infection can occur. A single oral dose of streptomycin can enhance susceptibility of laboratory animals to challenge by *Salmonella* spp. by at least 100 000-fold [4]. Another important and growing area of concern is the effect of antibiotics on the colonisation resistance properties of the indigenous microbiota resulting in the emergence and spread of resistant strains between patients and the dissemination of resistance determinants between microorganisms [1]. Reid and Friendship [5] state that in 1998 the World Health Organization cited diarrhoeal diseases as the second most common cause of disability-adjusted life-years lost and of death (2.2 million). However, in many instances there is an essential requirement for the administration of antibiotics, and hence it is necessary to identify means of minimising the adverse effects of antibiotics whilst maximising their potential benefits. One method is to select for antimicrobial agents

* Corresponding author. Tel.: +44 1639 825100; fax: +44 1792 472466.
E-mail address: nsjl.plummers@virgin.net (S.F. Plummer).

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that do not disturb the microbial colonisation resistance, but this is not always possible.

Beneficial effects have been observed when probiotics have been used for the prevention and treatment of gastrointestinal disturbance [6,7]. Trials have shown the potential for the use of probiotics in the treatment of rotavirus infections, antibiotic-associated diarrhoea, traveller's diarrhoea, infantile diarrhoea, relapsing *Clostridium difficile* colitis, inflammatory bowel disease, irritable bowel syndrome, atopy in at-risk infants and chronic sinusitis [8–15].

For the purposes of this study, a cohort of *Helicobacter pylori*-infected patients receiving the triple therapy antibiotic treatment regimen was selected for investigation.

The aim was to determine the effects of probiotic supplementation during triple therapy on the composition of the intestinal re-growth population, looking both at numbers and types of microorganisms and on the incidence of antibiotic resistance in the intestinal microbiota.

2. Materials and methods

2.1. Subjects

One hundred and sixty-two patients infected with *H. pylori* were enrolled into a study at Addenbrooke's Hospital, Cambridge, UK. The *H. pylori* infection was verified by positive serology and histology by the Public Health Laboratory Service at Addenbrooke's Hospital. Patients provided written consent and had no other gastrointestinal disorders apart from peptic ulcers thought to be related to their *H. pylori* infection. None had received any antibiotics or been subject to any dietary intervention in 6 weeks prior to the study. Ethical approval was obtained from Cambridge Local Research Ethics Committee.

2.2. Trial design

The trial was a double-blind placebo-controlled study with all patients receiving antibiotics from days 1 to 7. One group of patients received the probiotic product (active group) from days 1 to 21 and the second group received the placebo product (placebo group) from days 1 to 21. Two consecutive faecal samples were tested prior to antibiotic therapy and statistical analysis indicated that the data could be pooled to provide day 1 results. A further sample was collected on day 7. Two consecutive faecal samples were obtained 4 weeks after completion of antibiotic therapy and these were pooled to provide day 35 results. The faecal samples were sealed in anaerobic bags and stored at −70 °C until tested.

2.3. Treatment

The patients received standard eradication therapy: amoxicillin (1 g twice a day (bd)), clarithromycin (500 mg bd) and lansoprazole (30 mg bd) for 7 days. For penicillin allergy, 400 mg metronidazole three times a day was substituted. The probiotic product (Cultech Ltd., Port Talbot, UK) comprised two strains of *Lactobacillus acidophilus* (CUL60 and CUL21) and two strains of *Bifidobacterium* spp. at a total of 2.5×10^{10} colony-forming units (CFU)/capsule, and the placebo comprised an inactive carrier (maltodextrin). Patients received one capsule daily. The probiotic strains used were sensitive to test antibiotics using the disk diffusion assay according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines [16].

2.4. Compliance

Of the 162 patients recruited, 7 were excluded for failing to provide the samples. The 155 remaining patients were randomly divided between the placebo group (79 patients) and the active group (76 patients).

2.5. General microbiological screen

Traditional microbiological methods were used to analyse the samples. On the basis of pilot screening studies (unpublished data), selective media and Gram staining of colony types were used to enable enumeration and differentiation of the faecal microbiotas. An anaerobic dilution series of the faecal samples was set up in pre-reduced Maximum Recovery Diluent (MRD; Oxoid Ltd., Basingstoke, UK). A modification of the Miles and Misra plate count technique [17] was used to plate $10 \times 10\,\mu L$ of appropriate dilutions onto the pre-reduced selective agars (all agars were obtained from Oxoid Ltd. unless otherwise stated): anaerobic blood (total anaerobes; bioMerieux, Basingstoke, UK); blood agar (total facultative anaerobes; bioMerieux); Wilkins–Chalgren agar (*Bacteroides* spp.); MacConkey No. 3 agar (MAC; enterobacteriaceae); Kanamycin Aesculin Azide agar (KAA; enterococci); Baird Parker agar (staphylococci); de Mann Rogosa Sharpe agar (MRS; *Lactobacillus* spp.); modified MRS agar (0.3% (w/v) sodium propionate, 0.2% (w/v) lithium chloride, 0.05% (w/v) cysteine hydrochloride and 5% (v/v) defibrinated sheep blood included; *Bifidobacterium* spp.); Rose Bengal Agar (yeasts); and ID2 Agar (*Candida albicans*; bioMerieux).

Anaerobic plates were incubated at 37 °C for 72 h and aerobic plates were incubated at 37 °C for 48 h. Organisms were identified by anaerobic/aerobic growth colony, Gram stain and API biochemical identification strips (bioMerieux). The results were expressed as the CFU per gram of dry weight of faecal material.

2.6. Antibiotic resistance analysis

The effects of the antibiotic therapy on the numbers of antibiotic-resistant enterococci and enterobacteriaceae in the faecal microbiotas pre and post antibiotic treatment were chosen for assessment in this study. KAA agar or MAC

agar containing a range of concentrations of amoxicillin or clarithromycin (0, 0.015, 0.06, 0.5, 1.0, 4.0, 8.0, 16.0, 32.0, 128.0 or 512.0 μg/mL) were used for enumeration of enterococci and enterobacteriaceae, respectively.

The samples were plated out using the modified Miles and Misra technique [17] (10 × 10 μL drops) and plates were incubated aerobically at 37 °C for 48 h (KAA agar) or 24 h (MAC agar). The breakpoints for enterococci/amoxicillin at a minimum inhibitory concentration (MIC) >8 μg/mL, for enterobacteriaceae/amoxicillin at a MIC >32 μg/mL, for enterococci/clarithromycin at a MIC >1 μg/mL and for enterobacteriaceae/clarithromycin at a MIC >8 μg/mL represented antibiotic resistance [16,18].

2.7. Statistical analysis

Statistics were performed using the SPSS v11.5 program (SPSS, Chicago, IL, USA). Within the same treatment group, two related samples from days 1/2 and days 35/36 were compared using the Wilcoxon signed-rank test to ensure that pooling of the replicates was feasible. No significant differences were detected between these two sets of replicates (except staphylococci at days 1/2; Table 1). The Wilcoxon signed-rank test was also used to compare related samples in each microbial population between days 1 and 7, days 7 and 35, and days 1 and 35. The non-parametric Mann–Whitney *U*-test was used to compare the unrelated median values (active and placebo) for each microbial population. A *P*-value of less than 0.05 was considered statistically significant. The McNemar test was used to compare antibiotic resistance between any two time points (days 1, 7 and 35) and $P \leq 0.05$ indicates that the proportions are not equal.

3. Results

In the placebo and active groups, the total bacterial numbers decreased during antibiotic therapy, with a small clinical significance ($P < 0.05$), and increased post therapy (Tables 1 and 2). There were no significant differences between the numbers at days 1 and 35.

3.1. Changes in the numbers of enterobacteria and enterococci

In the placebo group, the numbers of facultative anaerobes increased significantly between days 7 and 35, and the numbers of enterobacteria were significantly higher at day 35 than at day 1 ($P < 0.05$). No significant changes occurred in the numbers of enterococci and staphylococci in this group, although the numbers of enterococci decreased during antibiotic therapy (Table 1).

In the probiotic-supplemented group, the enterobacterial population decreased during therapy but then increased so that at day 35 the numbers were not significantly different from those at day 1 (Table 2).

3.2. Effects on the bacteroides population

The numbers of bacteroides in the placebo group decreased significantly between days 1 and 7 ($P < 0.05$), followed by a significant increase during the re-growth period so that at day 35 the numbers were not significantly different from day 1. In contrast, in the active group the numbers of bacteroides increased from days 7 to 35 ($P < 0.01$) so that the final numbers at day 35 were significantly higher than at day 1 ($P < 0.05$).

3.3. Changes in lactobacilli and bifidobacteria

The bifidobacterial population in both groups decreased in response to antibiotic therapy ($P < 0.0001$) and, despite increasing significantly between days 7 and 35, the numbers for both groups at day 35 were significantly lower than those at day 1 ($P < 0.0001$).

Table 1
Distribution of the intestinal microbiota in patients in the placebo group[a]

Population	*N*[b]	Day 1	Day 7	Day 35
Total bacterial count	55	10.4 (9.2–11.8)*	9.8 (7.2–11.8)**	10.4 (7.6–12.5)
Total facultative anaerobes	57	8.8 (<1.7–11.8)	8.1 (<1.7–11.8)**	9.0 (<1.7–11.7)
Enterobacteriaceae	58	7.7 (4.0–11.4)*	5.1 (<1.7–10.2)**	8.6 (<1.7–10.4)†
Enterococci	59	6.3 (<1.7–11.4)	4.0 (<1.7–10.3)	6.7 (<1.7–10.9)
Staphylococci[c]	54	4.7 (<1.7–10.3), 4.0 (<1.7–10.0)	4.5 (<1.7–9.4)	5.3 (<1.7–9.5)
Total anaerobes	48	10.3 (9.2–11.8)	9.8 (7.2–11.4)**	10.4 (7.3–12.5)
Bacteroides	60	9.9 (<1.7–11.0)*	9.0 (<1.7–11.2)**	10.0 (<1.7–10.9)
Bifidobacteria	63	9.1 (<1.7–11.6)*	1.7 (<1.7–11.6)**	4.7 (<1.7–12.5)†
Lactobacilli	54	8.3 (5.2–11.1)*	6.9 (<1.7–10.0)**	8.7 (5.9–11.8)
Yeasts	52	<1.7 (<1.7–7.4)*	4.8 (<1.7–8.2)**	2.3 (<1.7–7.2)
Candida albicans	61	<1.7 (<1.7–7.4)*	4.4 (<1.7–8.0)**	2.2 (<1.7–5.8)†

Statistical analysis using SPSS v11.5 statistical package: the Wilcoxon signed-rank test comparison between sample collection days: *$P \leq 0.05$, day 1 compared with day 7; **$P \leq 0.05$, day 7 compared with day 35; †$P \leq 0.05$, day 1 compared with day 35.

[a] Data given as median (minimum–maximum) $\log_{10}$ colony-forming units (CFU)/g dry weight of faeces.

[b] Number of patients harbouring microbial population.

[c] There were significant differences between the medians of two samples before therapy (day 1), therefore the results from these samples could not be merged to provide day 1 results.

Table 2
Distribution of the intestinal microbiota in patients in the active group[a]

Population	N[b]	Day 1	Day 7	Day 35
Total bacterial count	55	10.4 (9.5–13.2)*	10.1 (<1.7–12.0)**	10.4 (8.2–11.7)
Total facultative anaerobes	56	8.6 (4.0–11.6)	8.0 (<1.7–12.0)	8.6 (6.7–12.0)
Enterobacteriaceae	56	7.8 (<1.7–11.5)*	5.5 (<1.7–9.8)**	8.1 (<1.7–9.8)
Enterococci	57	6.3 (<1.7–9.9)	4.7 (<1.7–10.6)	7.0 (<1.7–9.0)
Staphylococci	58	4.9 (<1.7–11.6)	<1.7 (<1.7–11.6)	5.0 (1.6–9.3)
Total anaerobes	50	10.4 (9.4–13.2)	10.1 (7.7–11.2)**	10.4 (6.7–11.7)
Bacteroides	57	9.9 (5.7–11.6)	9.9 (<1.7–11.1)**	10.2 (<1.7–11.4)†
Bifidobacteria	62	9.5 (<1.7–12.4)*	<1.7 (<1.7–10.7)**	7.3 (<1.7–10.6)†
Lactobacilli	53	8.2 (5.9–10.6)	6.9 (<1.7–11.2)	8.5 (5.8–10.0)
Yeasts	51	<1.7 (<1.7–7.7)*	4.5 (<1.7–7.9)**	<1.7 (<1.7–6.2)
Candida albicans	62	<1.7 (<1.7–7.4)*	<1.7 (<1.7–7.5)	2.2 (<1.7–7.9)†

Statistical analysis using SPSS v11.5 statistical package: the Wilcoxon signed-rank test comparison between sample collection days: $^{*}P \leq 0.05$, day 1 compared with day 7; $^{**}P \leq 0.05$, day 7 compared with day 35; $^{\dagger}P \leq 0.05$, day 1 compared with day 35.
[a] Data given as median (minimum–maximum) $\log_{10}$ colony-forming units (CFU)/g dry weight of faeces.
[b] Number of patients harbouring microbial population.

The lactobacillus population of the placebo group decreased significantly between days 1 and 7, but then increased ($P < 0.01$) so that at day 35 the numbers were comparable with those at day 1 (Table 1). The numbers of lactobacilli in the probiotic group decreased during antibiotic therapy and increased again post treatment, but none of these changes was statistically significant (Table 2).

3.4. The effects of antibiotics on the yeast component of the microbiota

Although the numbers of yeast increased in both groups during antibiotic therapy, in the placebo group this was associated with a significant increase in the number of *C. albicans*; a similar increase did not occur among the patients receiving the probiotic supplement. At day 35, the numbers of *C. albicans* in both groups were significantly higher than at day 1 (Tables 1 and 2).

3.5. Comparison of the components of the microbiotas of the two groups

When the microbiotas of the two groups were compared (Table 3), the numbers of total facultative anaerobes at day 35 in the active group were significantly lower than in the placebo group ($U = 1648$; $P = 0.031$). Similarly, the numbers of enterobacteriaceae in the active group were significantly lower than in the placebo group ($U = 1608$; $P = 0.014$). The number of *C. albicans* after antibiotic therapy in the placebo group was significantly higher than in the active group ($U = 1891$; $P = 0.049$), but by day 35 the numbers of yeasts were comparable in both groups. There were no significant differences between the two treatment groups for any of the other microbial populations tested.

3.6. Antibiotic resistance

A very high level of indigenous antibiotic resistance was found among the enterobacteriaceae in this cohort of patients, which made it very difficult to make any assessment of changes in the antibiotic resistance profiles. There was no decrease in resistance in response to probiotic supplement action, but the indigenous resistance levels were too high to determine whether the probiotics had registered any impact.

The development of resistance to amoxicillin and clarithromycin between days 1 and 35 among the enterococcal population of patients in the two groups is shown in Tables 4 and 5, respectively. At day 35 in the placebo group, the number of patients expressing antibiotic resistance within the enterococcal population was significantly higher ($P \leq 0.05$) than the number in the initial population at all antibiotic concentrations up to 32.0 μg/mL (Table 4). At the highest concentrations of amoxicillin (128 and 512 μg/mL), comparison of days 1 and 35 showed no significant differences in the levels of antibiotic resistance in the enterococcal population.

However, for the probiotic-supplemented group at all amoxicillin concentrations, there was no significant increase in the number of patients carrying antibiotic-resistant enterococci between days 1 and 35.

Table 3
Comparison of microbial populations among the placebo and active groups

Population	Day 7		Day 35	
	CFU/g[a]	*P*-value[b]	CFU/g[a]	*P*-value[b]
Total facultative anaerobes				
Placebo	8.1	0.598	9.0	0.031
Active	8.0		8.6	
Enterobacteriaceae				
Placebo	5.1	0.983	8.6	0.014
Active	5.5		8.1	
Candida albicans				
Placebo	4.4	0.049	2.2	0.815
Active	<1.7		2.2	

[a] Data given as median $\log_{10}$ colony-forming units (CFU)/g dry weight of faeces.
[b] According to Mann–Whitney *U*-test.

Table 4
Number of patients developing amoxicillin resistance within the faecal enterococcal population between days 1 and 35

Amoxicillin (μg/mL)	Number of patients	*P*-value[a]
Placebo group		
4.0	13	0.049
8.0[b]	12	0.035
16.0	10	0.012
Active group		
4.0	9	N.S.
8.0[b]	11	N.S.
16.0	6	N.S.

N.S., no significant difference.
[a] According to McNemar test.
[b] The breakpoint for enterococci:amoxicillin at a minimum inhibitory concentration (MIC) >8 μg/mL represented antibiotic resistance.

Table 5
Number of patients developing clarithromycin resistance within the faecal enterococcal population between days 1 and 35

Clarithromycin (μg/mL)	Number of patients	*P*-value[a]
Placebo group		
0.5	17	<0.001
1.0[b]	20	<0.001
4.0	29	<0.001
Active group		
0.5	7	N.S.
1.0[b]	14	0.013
4.0	22	0.001

N.S., no significant difference.
[a] According to McNemar test.
[b] The breakpoint for enterococci:clarithromycin at a minimum inhibitory concentration (MIC) >1 μg/mL represented antibiotic resistance.

With clarithromycin, in the placebo group there was a significant development of resistance ($P \leq 0.001$) at concentrations near to the resistance breakpoint, which was not seen in the probiotic group. However, significant resistance ($P = 0.001$) developed in both groups to the same extent at higher clarithromycin concentrations (Table 5).

4. Discussion

Administration of antibiotics often causes disturbances in the normal intestinal microbiota [19,20]. In the present study, the total bacterial and total facultative anaerobe population results indicate that despite the probiotic supplement the microbiotas of both the placebo and active groups were susceptible to the effects of the antibiotics administered to eradicate *H. pylori*. It appeared that there was recovery of the majority of the components of the microbiota post antibiotic therapy, with no significant difference between days 1 and 35. However, the noticeable difference occurred with the enterobacterial component of the placebo group, which was subject to disturbance, suggesting that supplementation with probiotics had impacted on the intestinal microbiota, resulting in less disruption of the compositional balance for the active group.

Madden et al. [21] found a significant increase in the facultative anaerobe component of the microbiota between days 1 and 27 in placebo group with amoxicillin, metronidazole and lansoprazole treatment. When probiotics were given after antibiotics, numbers decreased significantly between days 7 and 27 back to the starting levels.

The eradication therapy did not significantly disrupt the total anaerobe population from days 1 to 35 (Tables 1 and 2), which contrasts with the results of other studies where anaerobes were suppressed [18,22]. Adamsson et al. [18,23] found that the total anaerobic microbiota was strongly suppressed in *H. pylori* patients (amoxicillin and metronidazole combination (OAM) group or clarithromycin and metronidazole (OCM) group), although the effect was most pronounced in the OCM group. Amoxicillin as a single agent causes only minor disturbances, but in some studies the anaerobic microbiota has been found to be disrupted due to metronidazole [23,24].

It is also interesting that despite the sensitivity of the probiotic organisms to antibiotics, no significant changes were observed for the total *Lactobacillus* numbers in the probiotic-supplemented group—an observation not recorded for the placebo group. However, the antibiotic sensitivity of the bifidobacteria was apparent in both groups, as also observed by Adamsson et al. [18] and Buhling et al. [25].

Although there was no significant change in total numbers of yeast between days 1 and 35 in the placebo group, the number of *C. albicans* increased significantly ($P < 0.01$). This finding contrasts with the study of Buhling et al. [25], who found that the numbers both of yeast and *C. albicans* in patients with *H. pylori* returned back to the starting levels after 4 weeks.

The very high levels of antibiotic resistance among the enterobacteriaceae in this cohort of patients made any assessment of changes (increases) in resistance post therapy very difficult, but the extent of antibiotic resistance might have been related to the significantly lower numbers of enterobacteria seen in the active group patients compared with the placebo group at day 35. Working with a similar cohort, Stark et al. [22] observed overgrowth by amoxicillin-resistant enterobacteria post antibiotic therapy.

Antibiotic resistance among the enterococci was significantly higher in the placebo group than in the probiotic group post therapy in this study, suggesting that the probiotics had in some way modulated the composition of the re-growth population. It is known that bacteria have an energy requirement to achieve antibiotic resistance [26], either owing to chromosomal alterations (e.g. target site alterations) or owing to the use of accessory elements (such as enzymes and antibiotic efflux pumps). Such energy requirements could affect the growth kinetics of the bacteria, but the antibiotic resistance provides a competitive advantage over the antibiotic-sensitive strains, enabling their survival. The energy costs involved in the mechanisms of resistance for the bacteria in this study are unknown, but it is possible that the additional challenge to these ‘energy-depleted’ bacteria

caused by the daily supplement of probiotic bacteria could be too great to enable their domination and hence this could account for the lower incidence of antibiotic resistance among the active group patients.

From this study, it would appear that daily supplementation with viable probiotic bacteria during and post antibiotic therapy reduces the extent of disruption to the intestinal microbiota as well as the incidence and total numbers of antibiotic-resistant strains in the re-growth population.

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