

***In Vitro* Studies on Colonization Resistance of the Human Gut Microbiota to *Candida albicans* and the Effects of Tetracycline and *Lactobacillus plantarum* LPK**

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Abstract

An anaerobic three-vessel continuous-flow culture system, which models the three major anatomical regions of the human colon, was used to study the persistence of *Candida albicans* in the presence of a faecal microbiota. During steady state conditions, overgrowth of *C. albicans* was prevented by commensal bacteria indigenous to the system. However antibiotics, such as tetracycline have the ability to disrupt the bacterial populations within the gut. Thus, colonization resistance can be compromised and overgrowth of undesirable microorganisms like *C. albicans* can then occur. In this study, growth of *C. albicans* was not observed in the presence of an established faecal microbiota. However, following the addition of tetracycline to the growth medium, significant growth of *C. albicans* occurred. A probiotic *Lactobacillus plantarum* LPK culture was added to the system to investigate whether this organism had any effects upon the *Candida* populations. Although *C. albicans* was not completely eradicated in the presence of this bacterium, cell counts were markedly reduced, indicating a compromised physiological function. This study shows that the normal gut flora can exert 'natural' resistance to *C. albicans*, however this may be diminished during antibiotic intake. The use of probiotics can help fortify natural resistance.

Introduction

A diverse collection of microorganisms colonizes the human gastrointestinal tract, the majority of which reside in the colon (Steer *et al.*, 2000). More than 500 culturable species of bacteria make up this gastrointestinal microbiota (Moore and Holdeman, 1974; Suau *et al.*, 1999). Bacterial

metabolism in the intestine results in the production of certain physiologically beneficial compounds, for example short chain fatty acids. One important function of the indigenous flora is their action as a barrier against invasion by other microbes, such as pathogens. This natural phenomenon is known as 'colonization resistance'.

Yeasts such as *Candida albicans*, are also part of the normal microflora but cell counts do not normally exceed 10⁴⁻⁵ cfu/g faeces (Bernhardt *et al.*, 1995; Bernhardt and Knoke, 1997). *C. albicans* is the most common cause of candidiasis and is responsible for up to 10% of all nosocomial blood infections (Beck-Sague *et al.*, 1993). Candidiasis is common in the immunocompromised person and is associated with high morbidity in HIV-1 patients (McCarthy 1992). Overgrowth of *C. albicans* may not occur in the presence of a normal healthy microbiota, and it is feasible to support a role for probiotic intestinal bacteria in the prevention of candidiasis, including long term persistence by the yeast (Wagner *et al.*, 1997; Balish and Wagner, 1998). However, the specific bacteria involved in colonization resistance and the mechanisms of resistance have yet to be elucidated.

Disturbances to the microbial ecosystem within the gut may result in reduced colonization resistance (Edlund and Nord, 1991), and hence *Candida* overgrowth. This is likely to be undesirable since *Candida* spp. have been implicated as exerting a possible role in the development of disorders such as Irritable Bowel Syndrome (IBS) and recurrent vaginal thrush (Middleton *et al.*, 1992). Factors which may result in alterations to the normal gut flora include antibiotic usage (Bernhardt *et al.*, 1995; Bernhardt and Knoke, 1997; Mendall and Kumar, 1998). The use of oral antibiotics, especially when administered over a period of time, causes the elimination of a substantial proportion of the normal microbiota of the gastrointestinal tract. Hence, numbers of bacteria that would ordinarily compete with other organisms for nutrients and mucosal epithelial cellular receptor sites is greatly reduced, perhaps facilitating an excessive growth of yeasts.

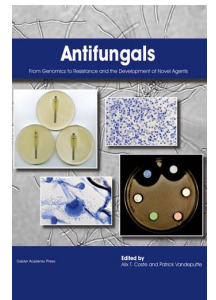
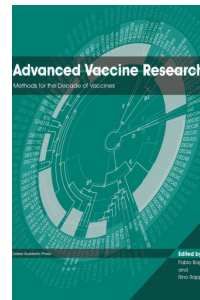
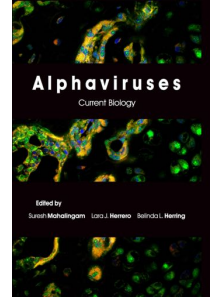
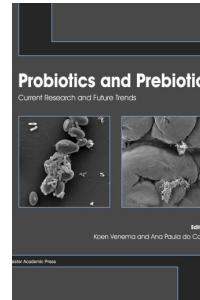
Probiotics, prebiotics and synbiotics are microflora management tools which have received much recent scientific interest for the prophylactic treatment or symptom relief of gastrointestinal complaints, such as inflammatory bowel disease, irritable bowel syndrome, antibiotic associated diarrhoea and candidiasis (Balish and Wagner, 1998; Collins and Gibson, 1999; Steer *et al.*, 2000; Molin, 2001; Marteau *et al.*, 2002). Amongst these strategies, probiotics have received the most attention and supportive scientific evidence. Probiotics are live microbial food supplements which beneficially effect host health and are generally members of the lactobacilli or bifidobacteria

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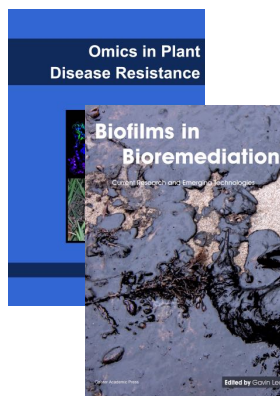
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(Fuller, 1989). Although their mechanisms of action have not been fully defined for specific disease states, they are thought to include pathogen inhibition through the production of short chain fatty acids and anti-microbial compounds (*i.e.* bacteriocins), stimulation of the immune system in a non-inflammatory manner, and competition for nutrients and mucosal adhesion sites with pathogenic microorganisms (Reid, 1999; Vaughan *et al.*, 1999; Fooks and Gibson, 2002). *Lactobacillus plantarum* has been shown to adhere to human intestinal cell lines and to produce a range of bacteriocin-like molecules, some of which are active against yeasts and fungi (Adlerberth *et al.*, 1996; Ström *et al.*, 2002).

In this study, a three-vessel continuous-culture system was used to investigate the inhibitory effects of the human gut microbiota on *C. albicans*. Tetracycline is a broad-spectrum antibiotic that has long been used to treat a wide range of infections. The aims of the experiment were to determine, in the steady state, whether the normal gut flora could exert colonization resistance to *C. albicans*. Tetracycline was then incorporated into the culture growth medium and its effects determined. A probiotic bacterium, *Lactobacillus plantarum* LPK was also introduced into the system to investigate whether its activities had any effect upon persistence of the yeast.

Experimental Procedures

Candida albicans

Candida albicans was routinely grown on Sabouraud Dextrose (SD) agar aerobically at 37°C. Stock cultures were stored at -70°C in Microbank® cryogenic storage tubes.

Preparation of *C. albicans* Inoculum

C. albicans was streaked onto SD agar and a single colony inoculated into 10ml SD broth. After aerobic incubation overnight at 37°C, 1ml was then used to inoculate 100ml of SD broth. Following overnight incubation, the culture was centrifuged at 10,000g, the supernatant decanted and the precipitate re-suspended in 10ml sterile phosphate buffered saline (Oxoid). The suspension was then re-centrifuged and the precipitate re-suspended in a further 10ml phosphate buffered saline. The suspension was serially diluted, plated onto SD agar and incubated aerobically at 37°C for 48 hours, to determine cell numbers.

Lactobacillus plantarum LPK

L. plantarum LPK strain, was originally isolated from the faeces of a healthy human volunteer and characterized by sequencing the entire 16S rRNA gene. A rifampicin resistant variant of *L. plantarum* LPK was selected by successive overnight, anaerobic growth in MRS broth (Oxoid) containing increasing concentrations of rifampicin (Sigma) from 0.0001 µg/ml to 100 µg/ml. Briefly, 100 µl of *L. plantarum* LPK overnight culture was used to inoculate 10 ml of MRS broth containing 0.0001 µg/ml rifampicin. The culture was incubated overnight anaerobically and 100 µl used to inoculate 10 ml of MRS broth containing 0.001 µg/ml. This culture was then incubated overnight. The process was repeated until growth was observed in MRS broth

containing 100 µg/ml rifampicin. A single colony of the rifampicin resistant *L. plantarum* LPK was grown up on MRS agar containing 100 µg/ml rifampicin. Stock cultures were prepared in Microbank® cryogenic storage tubes and stored at -70°C.

L. plantarum LPK Inoculum

A pure *L. plantarum* LPK culture was used to inoculate 10 ml pre-reduced MRS broth + 100 µg/ml rifampicin. Following anaerobic incubation overnight, the culture was centrifuged at 10,000g a total of three times, with re-suspension of the cell pellet in 10ml sterile phosphate buffered saline (Oxoid), to ensure complete washing. The suspension was serially diluted and plated onto Rogosa agar and Rogosa + 100 µg/ml rifampicin agar plates to enumerate the culture.

In Vitro Model of the Human Colonic Microbiota

The continuous culture system employed consisted of three culture vessels, V1, V2, and V3, and was designed to represent the three major regions of the human colon; proximal, transverse and distal, respectively (Figure 1). The system has been previously validated using gut contents from sudden death victims obtained at autopsy (Macfarlane *et al.*, 1998). Each vessel was maintained under anaerobic conditions, magnetically stirred and temperature controlled (37°C). The pH of the culture vessels was automatically controlled, and set to resemble that of the different colonic regions: pH 5.5, 6.2 and 6.9, respectively. The culture medium had the following constituents (g/l), dissolved in distilled water (Macfarlane *et al.*, 1998): Lintner's starch (BDH Ltd.), 5.0; pectin (citrus), 2.0; guar gum, 1.0; mucin (porcine gastric type III), 4.0; xylan (oatspelt), 2.0; arabinogalactan (larch wood), 2.0; inulin, 1.0; casein (BDH Ltd.), 3.0; peptone water, 5.0; tryptone, 5.0; bile salts No. 3, 0.4; yeast extract, 4.5; FeSO₄.7H₂O, 0.005; NaCl, 4.5; KCl, 4.5; KH₂PO₄, 0.5; MgSO₄.7H₂O, 1.25; CaCl₂.6H₂O, 0.15; NaHCO₃, 1.5; cysteine, 0.8; hemin, 0.05; Tween 80, 1.0. The medium was autoclaved at 121°C for 15 minutes and placed under nitrogen gas whilst hot. To inoculate the system, fresh faecal samples were obtained from healthy human subjects who reported no antibiotic usage during the previous two years and had no history of gastrointestinal disorder. A 10% (w/v) slurry was prepared using anaerobic phosphate buffered saline and approximately 100ml added to each culture vessel. Growth medium was pumped into V1 which subsequently fed V2 and V3, thereby rendering V1 a carbohydrate-rich environment with nutrient concentrations decreasing successively in vessels 2 and 3, simulating the natural conditions of the colon. Tetracycline (Sigma) was aseptically added to the culture medium at a concentration of 1g/l. The retention time of the culture system was calculated as the reciprocal of the dilution rate, and determined as 50.7 ± 2.5 hours, (mean±SD, n = 3); 67.5 ± 6.3 hours, (mean ± SD, n = 4) and 61.6 ± 5.1 hours (mean ± SD, n = 4) for gut models 1, 2 and 3 respectively.

Gut Model Dosing Regime

On day 18 after inoculation, 1 ml of *C. albicans* at 8.08 ± 0.05 log₁₀ cfu/ml was aseptically added into vessel 1.

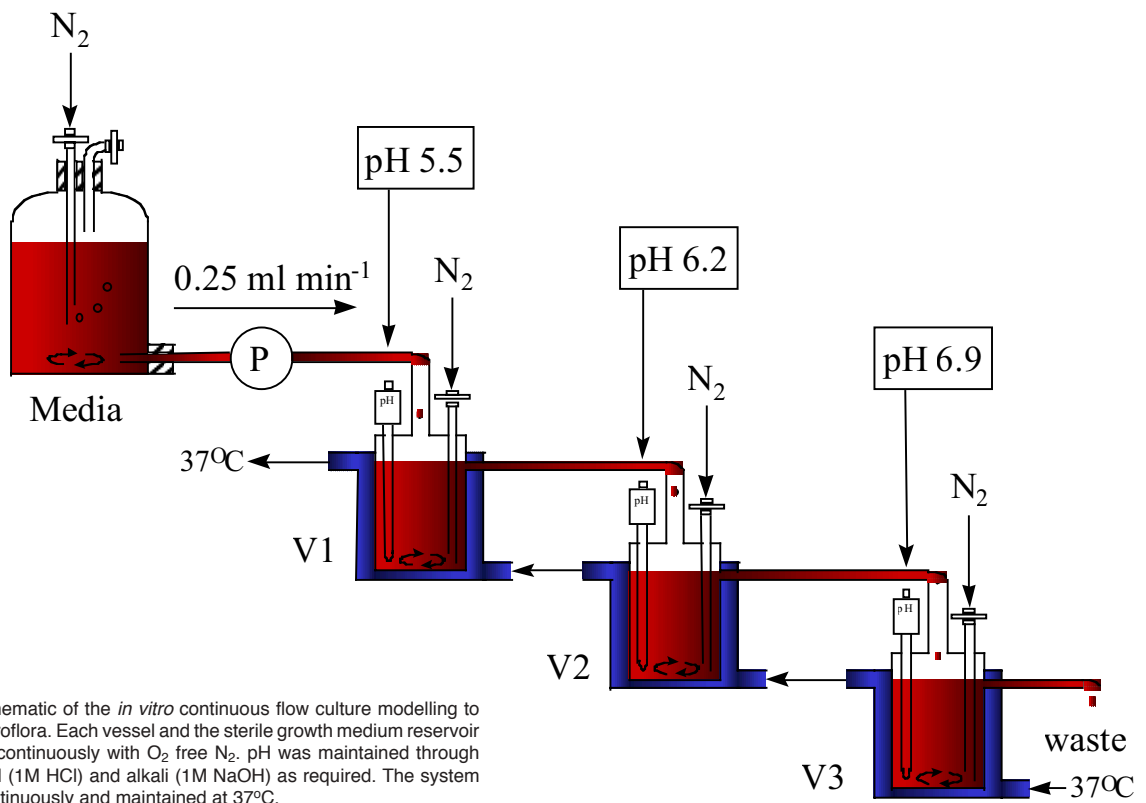


Figure 1. A schematic of the *in vitro* continuous flow culture modelling to human gut microflora. Each vessel and the sterile growth medium reservoir were sparged continuously with O_2 free N_2 . pH was maintained through addition of acid (1M HCl) and alkali (1M NaOH) as required. The system was stirred continuously and maintained at 37°C .

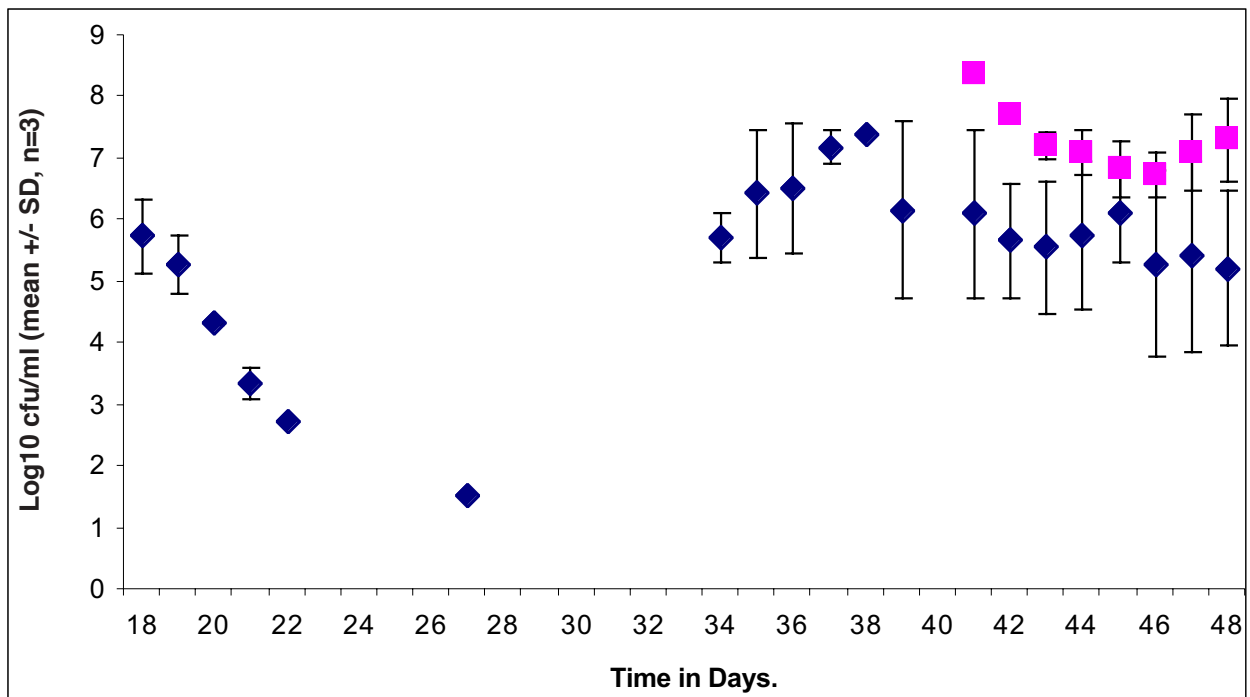


Figure 2. Population levels of *C. albicans* (◆) and *L. plantarum* LPK (■) in vessel one of the gut models are expressed as $\text{log}_{10} \text{ cfu/ml} \pm$ standard deviation ($n = 3$). The gut models were dosed with *C. albicans* on days 18 and 34, tetracycline was introduced on day 29 and the probiotic strain *L. plantarum* LPK on day 41.

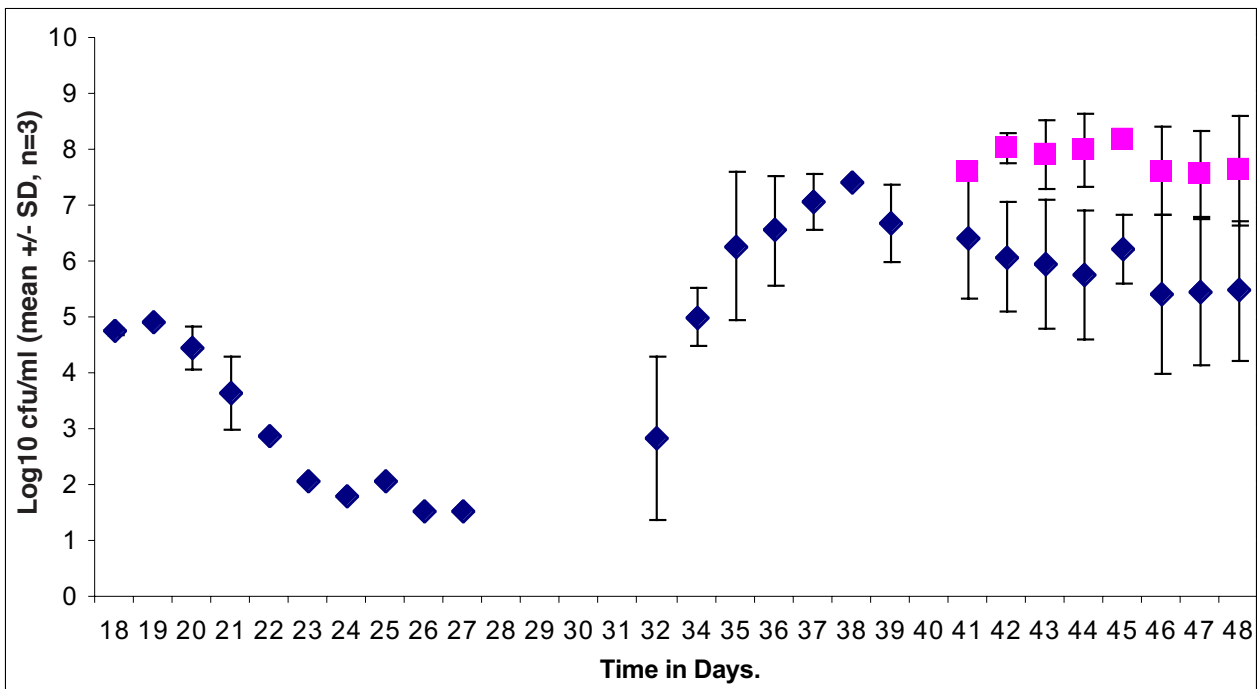


Figure 3. Population levels of *C. albicans* (◆) and *L. plantarum* LPK (■) in vessel two of the gut models are expressed as log₁₀ cfu/ml ± standard deviation (n = 3). The gut models were dosed with *C. albicans* on days 18 and 34, tetracycline was introduced on day 29 and the probiotic strain *L. plantarum* LPK on day 41.

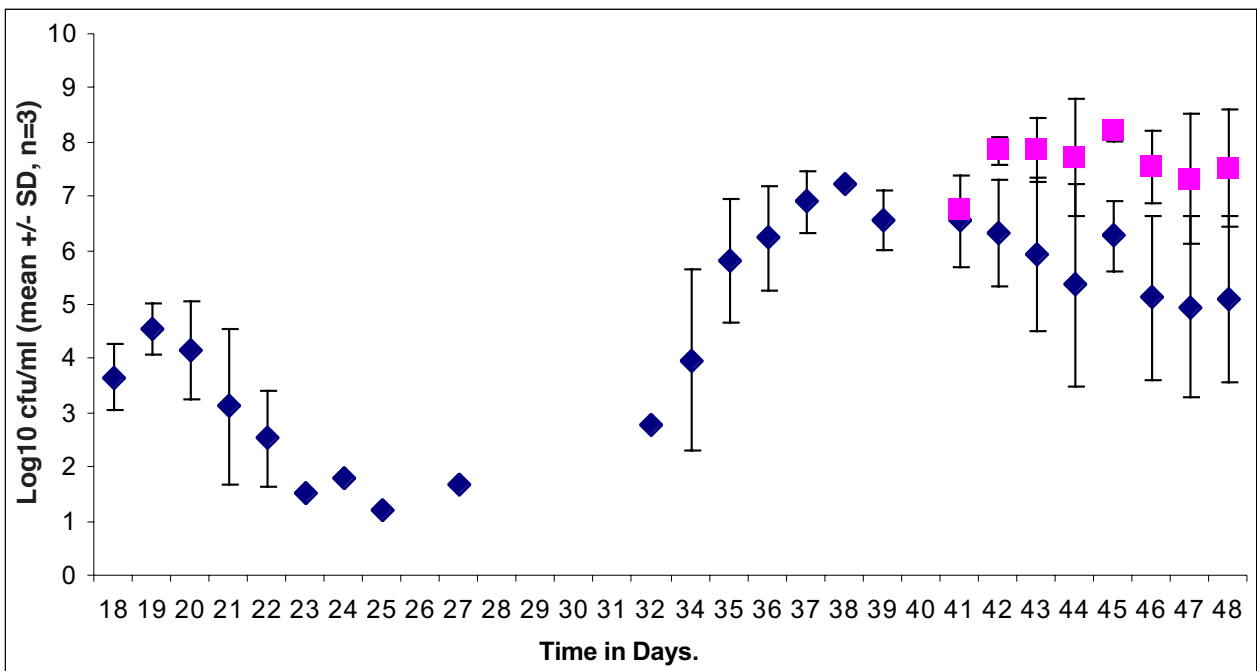


Figure 4. Population levels of *C. albicans* (◆) and *L. plantarum* LPK (■) in vessel three of the gut models are expressed as log₁₀ cfu/ml ± standard deviation (n = 3). The gut models were dosed with *C. albicans* on days 18 and 34, tetracycline was introduced on day 29 and the probiotic strain *L. plantarum* LPK on day 41.

Samples were removed from the system at regular intervals and plated onto SD agar until day 27. Total bacterial counts were also performed on days 18 and 27. Tetracycline (1 g/l) was added to the medium on day 29 and a total bacterial count carried out on day 32. On day 34, 1 ml of *C. albicans* at $8.35 \pm 0.31 \log_{10}$ cfu/ml was aseptically added into vessel 1. A total bacterial count was performed on day 36 and *C. albicans* enumerated at regular intervals between days 32 and 39. On day 41, total bacterial counts were again carried out and 5 ml *L. plantarum* LPK at $10.62 \pm 0.31 \log_{10}$ cfu/ml was then aseptically added into vessel 1. Enumeration of lactobacilli was performed at regular intervals until day 48 using Rogosa agar and Rogosa + Rifampicin (100 μ g/ml) agar. The number of *C. albicans* within the system was also monitored using Sabouraud Dextrose agar. A final total bacterial count was performed on day 48. The experiment was repeated three times with triplicate plating at each time point.

Bacteriology

A sample was taken from the faecal slurry, and at each subsequent sampling time from each culture vessel. The samples were serially diluted 10-fold with pre-reduced half-strength peptone water. Appropriate dilutions were plated, in triplicate, onto various selective agars. Table 1 shows the selective growth agars used to enumerate members of the human faecal microbiota present in the gut model. For anaerobic bacteria all media were pre-reduced under anaerobic conditions (N_2 , CO_2 and H_2) and maintained at 37°C (Wang and Gibson, 1993; Tuohy *et al.*, 2002). Anaerobic plates were incubated for approximately 96 hours, and aerobic plates for approximately 24 hours before enumeration. Colonies displaying different morphologies were chosen from plates and culture identities confirmed as described by Gibson *et al.* (1995).

Table 1. Selected growth media and bacterial groups enumerated.

Selective Growth Media ^a	Dilutions plated	Target Group ^b
Nutrient agar	$10^{-0} - 10^{-5}$	Total aerobes
MacConkey agar No.3	$10^{-0} - 10^{-4}$	Coliforms
Wilkins-Chalgren agar	$10^{-4} - 10^{-7}$	Total anaerobes
Bacteroides agar ^c	$10^{-0} - 10^{-7}$	<i>Bacteroides</i> spp.
Beerens agar (BA) ^d	$10^{-0} - 10^{-7}$	<i>Bifidobacterium</i> spp.
Azide/Crystal violet agar ^e	$10^{-0} - 10^{-4}$	<i>Enterococcus</i> spp.
Rogosa agar	$10^{-0} - 10^{-7}$	<i>Lactobacillus</i> spp.
SD agar ^f	$10^{-0} - 10^{-5}$	<i>C. albicans</i>
Clostridia agar ^g	$10^{-3} - 10^{-6}$	<i>Clostridium</i> spp.
Rogosa agar + Rifampicin (100 μ g/ml)	$10^0 - 10^5$	<i>L. plantarum</i> LPK Rif ^g

^a All agar media purchased from Oxoid.

^b Target bacterial group of selective growth media, colonies being described through growth requirements and morphotype.

^c Brucella Medium Base (Oxoid) + 7.5mg/l vancomycin, 7.5mg/l kanamycin, 5 mg/L hemin, 50ml/l Laked Horse Blood and 200 μ l vitamin K solution for bacteroides.

^d Columbia agar with agar (5 g/l), glucose (5 g/l), cysteine HCl (0.5 g/l) and propionic acid (5 ml/l) to pH 5 (Beerens, 1990).

^e Azide agar (Oxoid) + 50ml/l Laked Horse Blood and 4ml/l crystal violet at 0.05% (v/v) for gram-positive cocci.

^f Sabouraud Dextrose (SD) agar (Mycological peptone (Oxoid) 10g/l, Glucose 40g/l, Agar technical No. 3 (Oxoid) 15 g/l, chloramphenicol 0.4 g/l and cycloheximide 0.05g/l) for *C. albicans*.

^g Reinforced Clostridial agar with novobiocin (8 mg/l) and colistin (8 mg/l).

Statistical analysis

Paired t-tests were used to determine the significance of differences in bacterial populations within the gut models at different time points. A p-value of less than 0.05 was considered as significant.

Results

Survival of *C. albicans* in the Human Colonic Microbiota Model

Figures 2-4 show the ability of *C. albicans* to be maintained in the three different vessels of the colonic microbiota model between days 18 and 27. Following the addition of 1ml *C. albicans* at $\log 8.08 \pm 0.05 \log_{10}$ cfu/ml into vessel 1 on day 18, the yeast cells were quickly lost from the microbiota. Numbers were reduced to the detection limit ($1.2 \log_{10}$ cfu/ml) within 5 days on day 23 in all three vessels. Since the rate of decrease in all three vessels was greater than the respective dilution rate, it can be concluded that the results obtained were not due simply to *Candida* being washed out of the system. By day 27, *C. albicans* was hardly detectable in all three vessels. Tetracycline was added to the growth medium of the triplicate gut models on day 29. This antibiotic had an effect upon bacterial population levels within the gut model systems (Tables 2-4). A small (non-significant) increase in *C. albicans* numbers was observed in vessels 2 and 3 of the gut models on day 32. On day 34, each gut model was inoculated with *C. albicans* at $8.35 \pm 0.31 \log_{10}$ cfu/ml directly into vessel 1. By day 36, *C. albicans* numbers had reached 6.53 ± 1.05 , 6.56 ± 0.97 and $6.25 \pm 0.96 \log_{10}$ cfu/ml respectively in vessels 1, 2 and 3.

Impact of Tetracycline on Microbiota Composition In Vitro

No significant differences in numbers of any of the bacterial groups enumerated were observed between days 18 and 27. On day 29, tetracycline (1 g/l) was introduced into the gut model growth medium. Tables 2-4 show the impact of the antibiotic on the population levels of major bacterial groups present in the system. For vessel 1, where the antimicrobial potency of the tetracycline would be expected to be greatest, numbers of all bacterial groups enumerated decreased dramatically. In particular, numbers of total anaerobes decreased significantly from $9.50 \pm 0.27 \log_{10}$ cfu/ml to $5.42 \pm 0.35 \log_{10}$ cfu/ml ($P=0.029$). Similarly, numbers of bifidobacteria and lactobacilli decreased significantly by more than 4 and 2 log units respectively. In vessel 2, numbers of all the bacterial groups were reduced by the addition of the antibiotic. In particular, numbers of total anaerobes and *Bacteroides* spp. decreased significantly from 9.66 ± 0.11 and $8.76 \pm 0.44 \log_{10}$ cfu/ml on day 27 to 6.13 ± 0.59 and $5.11 \pm 0.71 \log_{10}$ cfu/ml respectively on day 32. The addition of tetracycline to the gut model medium had a lesser effect on microbial populations in vessel 3. No significant differences were observed in numbers of any of the bacterial groups tested between days 27 and 32 for this region of the gut model, although *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp. and the lactobacilli were reduced. In contrast to the survivability of *C. albicans* in the gut models before antibiotic treatment, numbers of the yeast increased

Table 2. Populations of the major faecal bacterial groups present in vessel one of the gut models over the course of the study. Bacterial numbers are expressed as \log_{10} cfu/ml \pm standard deviation ($n = 3$). BDL is below detection limit. On day 41, total counts were taken before addition of *L. plantarum* LPK and counts of the probiotic strain taken 10 minutes after inoculation of the gut models with *L. plantarum* LPK*.

Day	18	27	32	36	41	48
Total Anaerobes	9.26 \pm 0.84	9.50 \pm 0.27	5.42 \pm 0.35	6.00 \pm 0.77	7.99 \pm 1.11	8.03 \pm 0.32
Total Aerobes	7.37 \pm 0.44	6.97 \pm 0.88	5.00 \pm 0.70	6.20 \pm 1.70	7.52 \pm 0.98	6.59 \pm 1.22
<i>Bacteroides</i> spp.	7.69 \pm 1.14	8.97 \pm 0.59	3.52	BDL	BDL	5.36
<i>Bifidobacterium</i> spp.	8.85 \pm 0.77	8.58 \pm 0.79	4.54 \pm 0.01	4.27 \pm 0.11	3.16 \pm 0.64	BDL
<i>Clostridium</i> spp.	7.25 \pm 1.48	7.58 \pm 1.34	BDL	BDL	8.56	8.53
<i>Lactobacillus</i> spp.	6.24 \pm 1.59	6.46 \pm 0.57	4.02 \pm 0.47	3.72 \pm 0.89	4.73 \pm 3.30	7.28 \pm 0.66
Gram + cocci	7.14 \pm 0.61	6.32 \pm 1.61	4.57 \pm 0.66	4.44 \pm 0.87	3.51 \pm 1.14	4.19 \pm 1.53
Coliforms	6.24 \pm 1.22	6.29 \pm 0.36	4.07 \pm 1.63	BDL	BDL	BDL
<i>C. albicans</i>	5.74 \pm 0.59	1.52	BDL	6.53 \pm 1.05	6.10 \pm 1.37	5.23 \pm 1.24
<i>L. plantarum</i> LPK					8.39 \pm 0.14*	7.31 \pm 0.68

up until day 38, and were maintained at a population level greater than 6 \log_{10} cfu/ml until day 41. Indeed, by day 36 *C. albicans* dominated the microbiota in vessels 1 and 2, while they constituted a significant proportion of the microorganisms in vessel 3.

Impact of the Probiotic Strain *L. plantarum* LPK on the Tetracycline Treated Gut Model and *C. albicans* Numbers
On day 41, each gut model was inoculated through vessel 1 with 5ml *L. plantarum* LPK at $10.62 \pm 0.40 \log_{10}$ cfu/ml. Initial population levels of *L. plantarum* LPK in vessels 1, 2 and 3 were 8.39 ± 0.14 , 7.63 ± 0.16 and $6.78 \pm 0.15 \log_{10}$ cfu/ml respectively 10 minutes after dosing the gut models. The probiotic strain was maintained at between 7.2 and 7.6 \log_{10} cfu/ml, with little variation between the three gut model vessels until the end of the experiment on day 48. In all three vessels, *L. plantarum* LPK was maintained at a population level greater than total *Lactobacillus* counts at the beginning of the experiment on days 18 and 27, and accounted for a sizable proportion of the total microbiota in each vessel between days 41 and 48 (about 1 in 10 total bacteria in vessels 1 and 2, and 1 in 100 total bacteria in vessel 3). The addition of *L. plantarum* LPK had a significant effect on levels of *C. albicans* in the gut models. In vessel 1, *C. albicans* decreased from $6.10 \pm 1.37 \log_{10}$ cfu/ml on day 41 to $5.23 \pm 1.24 \log_{10}$ cfu/ml on day 48 ($P=0.009$). In vessel 2, *C. albicans* numbers were reduced from $6.42 \pm 1.07 \log_{10}$ cfu/ml on day 41 to $5.49 \pm 1.25 \log_{10}$

cfu/ml on day 48 ($P=0.013$). Similarly, in vessel 3 a sizeable, although non-significant reduction (1.45 \log_{10} units) was observed upon the addition of *L. plantarum* LPK.

Discussion

The results obtained from these experiments support the hypothesis that the indigenous intestinal microbiota has the ability to suppress *C. albicans* in the colon, and that disruption to normal bacterial populations by tetracycline may result in *Candida* overgrowth. *C. albicans* was not able to colonize any of the three culture vessels of the gut model in the presence of a normal faecal flora. Moreover, the addition of *C. albicans* caused no significant alterations to bacterial cell counts. Upon addition to vessel 1 of the triplicate gut models, *C. albicans* numbers steadily decreased and were reduced to barely detectable levels 5 days after the original inoculation.

The addition of tetracycline to the growth medium significantly altered the composition of the indigenous microbiota, particularly in vessel 1, which received tetracycline directly from the medium reservoir. Total anaerobes, clostridia, bacteroides, bifidobacteria and lactobacilli were all reduced in number upon the addition of tetracycline. *Bifidobacterium* spp. were particularly affected, falling to below their detection limit by day 48 in all the vessels and in each replicate gut model. Total aerobes were not significantly decreased although gram-

Table 3. Populations of the major faecal bacterial groups present in vessel two of the gut models over the course of the study. Bacterial numbers are expressed as \log_{10} cfu/ml \pm standard deviation ($n = 3$). BDL is below detection limit. On day 41, total counts were taken before addition of *L. plantarum* LPK and counts of the probiotic strain taken 10 minutes after inoculation of the gut models with *L. plantarum* LPK*.

Day	18	27	32	36	41	48
Total Anaerobes	9.49 \pm 0.74	9.66 \pm 0.11	6.13 \pm 0.59	6.69 \pm 1.80	7.98 \pm 1.64	8.62 \pm 0.05
Total Aerobes	6.87 \pm 0.79	6.68 \pm 0.85	5.09 \pm 1.25	6.50 \pm 1.07	6.38 \pm 1.42	7.16 \pm 1.24
<i>Bacteroides</i> spp.	8.72 \pm 1.15	8.76 \pm 0.44	5.11 \pm 0.71	8.18 \pm ND	6.97 \pm 2.49	6.46 \pm 2.30
<i>Bifidobacterium</i> spp.	9.03 \pm 0.80	8.52 \pm 0.88	4.99 \pm 0.16	4.19 \pm 0.04	3.60 \pm 0.70	BDL
<i>Clostridium</i> spp.	7.98 \pm 1.79	7.73 \pm 1.51	4.98 \pm 0.30	5.80 \pm ND	5.07 \pm ND	8.48 \pm ND
<i>Lactobacillus</i> spp.	6.11 \pm 1.70	6.65 \pm 0.62	4.78 \pm 0.89	3.92 \pm 0.51	3.40 \pm 0.71	7.63 \pm 1.00
Gram + cocci	6.89 \pm 0.61	6.04 \pm 1.40	4.70 \pm 1.66	5.60 \pm 0.29	5.70 \pm 0.54	6.24 \pm 0.96
Coliforms	5.95 \pm 0.75	5.79 \pm 0.92	4.15 \pm 2.05	4.00 \pm ND	3.13 \pm ND	3.39 \pm ND
<i>C. albicans</i>	4.76 \pm 0.05	1.52 \pm ND	2.85 \pm 1.46	6.56 \pm 0.97	6.42 \pm 1.07	5.49 \pm 1.25
<i>L. plantarum</i> LPK					7.62 \pm 0.16*	7.64 \pm 0.98

Table 4. Populations of the major faecal bacterial groups present in vessel three of the gut models over the course of the study. Bacterial numbers are expressed as log₁₀ cfu/ml ± standard deviation (n = 3). BDL is below detection limit. On day 41, total counts were taken before addition of *L. plantarum* LPK and counts of the probiotic strain taken 10 minutes after inoculation of the gut models with *L. plantarum* LPK*.

Day	18	27	32	36	41	48
Total Anaerobes	9.10 ± 0.70	9.46 ± 0.22	9.42 ± 0.26	9.50 ± 0.37	9.59 ± 0.03	9.15 ± 0.02
Total Aerobes	6.76 ± 0.77	6.41 ± 0.84	7.71 ± 0.82	7.58 ± 1.14	7.72 ± 1.43	8.39 ± 0.55
<i>Bacteroides</i> spp.	8.72 ± 1.15	8.76 ± 0.44	5.11 ± 0.71	8.18 ± 0.71	6.97 ± 2.49	6.46 ± 2.30
<i>Bifidobacterium</i> spp.	8.76 ± 0.71	8.35 ± 1.15	5.66 ± 0.04	4.59 ± 0.76	3.80 ± 0.10	BDL
<i>Clostridium</i> spp.	7.54 ± 1.59	7.68 ± 1.49	6.78 ± 2.18	7.20 ± 3.01	6.48 ± 1.87	7.14 ± 3.17
<i>Lactobacillus</i> spp.	5.95 ± 1.64	6.50 ± 0.57	5.39 ± 0.42	4.31 ± 0.30	3.72 ± 0.77	7.50 ± 1.06
Gram + cocci	6.67 ± 0.62	5.77 ± 1.34	7.33 ± 1.47	6.34 ± 3.16	8.36 ± 0.57	8.05 ± 0.30
Coliforms	5.70 ± 0.61	5.54 ± 0.74	5.50 ± 0.66	4.66 ± 1.41	4.70 ± 1.10	5.84 ± 1.41
<i>C. albicans</i>	3.68 ± 0.63	1.70 ± ND	2.81 ± ND	6.25 ± 0.96	6.57 ± 0.84	5.12 ± 1.53
<i>L. plantarum</i> LPK					7.62 ± 0.16*	7.64 ± 0.98

positive cocci were affected, and coliforms were particularly sensitive. Following inoculation of the system with *C. albicans* on day 34, considerable growth was observed. In each of the three gut model vessels, *C. albicans* numbers continued to increase up to day 38, at which level they were maintained until the addition of the probiotic strain. Thus, it was clearly demonstrated that for the yeast to survive in the system it was necessary to disrupt the normal bacterial flora. Similar results have been described previously by Bernhardt *et al.*, (1995), who also used a continuous-flow culture system. It was also shown that the input of faecal flora to a continuous flow culture resulted in a reduction in yeast numbers (Bernhardt *et al.*, 1995). *Candida albicans* has been found to be eliminated from continuous flow faecal cultures at a similar rate to that of untreated animals (Kennedy *et al.*, 1988). Following oral yeast challenge, conventional mice were found to shed *C. albicans* within 48 hours and became free from detectable yeast within 32 days (Samonis *et al.*, 1994). However, after tetracycline treatment, yeast were found to colonize the gut within 48 hours and persist for up to 32 days.

The mechanisms by which *Candida* spp. is able to colonize the human gut remain poorly understood, although it is now well recognised that a healthy resident microbiota offers the best protection (Freter *et al.*, 1983; Kennedy and Volz, 1985; Kennedy *et al.*, 1987). Under normal circumstances, the indigenous microbiota form dense biofilms on the intestinal mucosal surfaces. Similarly, in the culture system used here thick layers of bacteria were present on the vessel walls. Biofilms are thought to be very important for maintaining the mucosal association of *C. albicans* at low levels. This is thought to occur as a result of bacteria out-competing the yeast for adhesion sites, and their production of inhibitory substances, such as organic acids and secondary bile acids, both of which have been seen to inhibit the association of *C. albicans* with intestinal mucosal tissues (Bernhardt and Knoke, 1997). It has not yet been established whether these inhibitors affect *Candida* adhesins, mucosal receptor(s), or both. Competition for substrates is a second major factor in determining microbial populations and each particular substrate will be utilised primarily by the species most efficient at metabolising that substrate (Freter *et al.*, 1983). Considering these factors it is easy to understand how *Candida* overgrowth may occur, *i.e.* usually following disruption of the gut flora.

Evidence was gained from these experiments to support a role for *L. plantarum* LPK as a probiotic strain with anti-*Candida* activities. The addition of this probiotic strain led to significant reduction in numbers of *C. albicans* in all three gut model runs. Moreover, *C. albicans* counts were reduced to levels within the normal range of about log 5 cfu/ml by day 48. Studies have shown that a number of other bacterial species are able to inhibit *Candida* growth (Balish and Wagner, 1998). Most have been performed on gnotobiotic rodents but *Escherichia coli*, lactobacilli, oral streptococci and bifidobacteria have all been shown to suppress *C. albicans* in the gastrointestinal tract in co-culture experiments. Probiotic strains of lactobacilli and bifidobacteria have even been shown to repress *C. albicans* numbers and the severity of candidiasis in immunodeficient gnotobiotic animals (Wagner *et al.*, 1997; Balish and Wagner, 1998). A variety of mechanisms have been proposed to explain the anti-*Candida* activities of such probiotic bacteria including nutritional competition, the production of anti-microbial compounds (bacteriocins as well as H₂O₂) targeting *Candida*, stimulation of phagocytes, increased intestinal peristalsis and intestinal epithelial cell renewal rates, alteration of pH and the production of an anaerobic oxidation-reduction potential (Balish and Wagner, 1998).

In conclusion, this study has shown that the normal gut flora can afford colonization resistance to *C. albicans*. This may be compromised by broad spectrum antibiotics however probiotics can help to maintain inhibitory effects towards the yeast. To help reduce the possibilities of *Candida* overgrowth during antimicrobial intake dietary modulation of the gut flora designed to enhance the protective capabilities should therefore be exploited.

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