



## Functional anatomy of the colonic bioreactor: Impact of antibiotics and *Saccharomyces boulardii* on bacterial composition in human fecal cylinders

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### ABSTRACT

Sections of fecal cylinders were analyzed using fluorescence in situ hybridization targeting 180 bacterial groups. Samples were collected from three groups of women ( $N=20$  each) treated for bacterial vaginosis with ciprofloxacin + metronidazole. Group A only received the combined antibiotic regimen, whereas the A/Sb group received concomitant *Saccharomyces boulardii* CNCM I-745 treatment, and the A.Sb group received *S. boulardii* prophylaxis following the 14-day antibiotic course. The number of stool cylinders analyzed was 188 out of 228 in group A, 170 out of 228 in group A/Sb, and 172 out of 216 in group A.Sb.

The colonic biomass was organized into a separate mucus layer with no bacteria, a 10–30  $\mu\text{m}$  broad unstirred transitional layer enriched with bacteria, and a patchy fermentative area that mixed digestive leftovers with bacteria. The antibiotics suppressed bacteria mainly in the fermentative area, whereas abundant bacterial clades retreated to the transitional mucus and survived. As a result, the total concentration of bacteria decreased only by one order. These effects were lasting, since the overall recovery of the microbial mass, bacterial diversity and concentrations were still below pre-antibiotic values 4 months after the end of antibiotic treatment. Sb-prophylaxis markedly reduced antibiotic effects and improved the recovery rates. Since the colon is a sophisticated bioreactor, the study indicated that the spatial anatomy of its biomass was crucial for its function.

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### Introduction

Antibiotics have changed microbiology profoundly in the last 100 years. However, although they can help to defeat infections, antibiotics can also devastate bacterial communities involved in the maintenance of normal body functions [1]. The well-reviewed clinical symptoms of antibiotic induced shifts in the microbiome of the colon are especially manifest and they can be extremely severe [6,18]. Despite a long history of problems, changes in the microbiota are poorly characterized and their interactions with colonic mucus are completely unknown. The studies prior to the 1970s

used culture methods, which are inadequate for the monitoring of polymicrobial communities. Nevertheless, they demonstrated that antibiotics massively altered the colonic microbiota. Subsequently, the development of molecular methods extended our perception of the diversity and abundance of colonic microorganisms [12]. They were, however, mainly focused on identification of the microbiota involved because of the high costs related to animal [9] and single patient [4] studies.

We have routinely used fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes for the monitoring of intestinal [15] and vaginal [16] microbiota in the Outpatient Clinic for Polymicrobial Infections and Biofilms of the Charité Hospital in Berlin, Germany since 2009. The colonic microbiota is visualized in paraffin sections of fixed stool-cylinders, which are collected by the patients with a weekly to monthly frequency, depending on the clinical aim. The spatial differences in bacterial growth in the

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center of the feces or on the mucus–feces interface provide information on the host microbial interactions during the process of colonic fermentation. Usually, each stool cylinder is evaluated with FISH probes for 6–8 of the most informative bacterial groups. However, the number of bacterial groups investigated can be increased to 300–400 depending on the question to be answered. This approach has shown diagnostic efficiency in patients with ulcerative colitis, Crohn's disease, irritable bowel syndrome and other gastrointestinal disorders. To date, we have investigated over 35,000 fecal cylinders.

A critical aspect in the evaluation of the colonic microbiota is the impact of concomitant antibiotic therapy, since both disease and antibiotics alter the colonic microbiota. Polymicrobial infections are also common in uro-vaginal disorders. Bacterial vaginosis (BV) is a local, non-lethal, persistent, extra-intestinal infection related to a *Gardnerella*-dominated biofilm. The disease occurs in 10–20% of sexually active women. The microbial diagnosis requires sophisticated culture-independent methods and is, in practice, deduced from indirect criteria, including clinical symptoms, pH and bacterial appearance in native or Gram-stained vaginal smears. The symptoms respond well to antibiotics, but recur in 60–70% of patients within weeks, leading to repeated antibiotic use. This particular group of patients then requires measures for monitoring and preventing colonic dysbiosis because of frequent antibiotic use. Therefore, we decided to intensify the surveillance of the colonic microbiota (intervals and number of microbial FISH probes) in a group of women treated with antibiotics for BV and evaluate the additional prophylactic use of *Saccharomyces boulardii* (*Sb*). *Saccharomyces boulardii* CNCM I-745 has been proven to be effective in the treatment of diarrhea [3] and is the only probiotic approved as a drug in most countries of the European Union [11].

## Materials and methods

### Patients and samples

Patients were considered eligible for the present study at the Charité University Hospital if they had persistent bacterial vaginosis (BV), and if treatment was indicated according to current guidelines CDC, 2010.

The diagnosis of BV in symptomatic women was confirmed using FISH in three consecutive urine samples collected monthly, which is an approach that has an excellent diagnostic accuracy compared to the Nugent score-based diagnosis, as described previously [19]. Biofilms were detected on desquamated vaginal epithelial cells that are abundantly present in urine sediments.

All subjects gave their informed consent. The collection of fecal and urine samples for FISH diagnosis of dysbiosis was approved by the ethics commission of the Charité University Hospital.

In this study, BV was treated with a combination of oral metronidazole ( $3 \times 400 \text{ mg day}^{-1}$ ) and ciprofloxacin ( $2 \times 500 \text{ mg day}^{-1}$ ) for 2 weeks. For prophylaxis with *Saccharomyces boulardii* CNCM I-745 [Perenterol®, Biocodex], one 250 mg capsule three times daily for 2 weeks was used.

Patients were randomly assigned to one of three groups ( $N=20$  each): women in Group A received the combined antibiotic regimen but no probiotics; women in Group A/*Sb* received prophylaxis with *S. boulardii* that was initiated concomitantly with antibiotic treatment; and women in Group A.*Sb* received *S. boulardii* following the 14-day antibiotic course.

Three consecutive stool samples were collected monthly prior to therapy. Two stool samples were collected weekly during antibiotic therapy on days 7 and 14, and four samples were collected

every 2 weeks after cessation of antibiotic treatment. Thereafter, 2–3 samples were collected monthly. A loss of a maximum of 3 samples for liquid stools (maximum of one in each period) was tolerated. However, liquid stools were uncommon in persons with a healthy gut and no patient was removed from the study for this reason, even during antibiotic treatment. This approach resulted in 9–12 stool samples per patient (days –60, –30, 0, 7, 14, 28, 42, 56, 70, 100, 130, and 160 as related to the start of antibiotic therapy).

The patients collected the stool samples themselves. The 4–10 mm long stool cylinders were punched-out from the stool using plastic drinking straws with an inside diameter of 3 mm. The drinking straws were pre-cut to 4 cm in length and handed out to the study participants together with 50 mL Falcon tubes filled with 20 mL Carnoy solution (6/6/1 vol. ethanol/glacial acetic acid/chloroform). The pieces of drinking straw with the stool inside were dropped immediately into the 50 mL Falcon tube, fixed in Carnoy solution for at least 24 h at room temperature and delivered to the laboratory within two weeks, as previously described [15].

### FISH

Colonic microbiotas were investigated using structure functional FISH analysis of Carnoy fixed and paraffin-embedded stool cylinders [15]. Multicolor fluorescence in situ hybridization (FISH) simultaneously using three differently stained FISH probes (C3 – orange, FITC-dobe – green, C5 – dark red) counterstained with DAPI to reveal DNA structures was performed on  $4 \mu\text{m}$  longitudinally cut sections of punched stool cylinders. Sections were placed on SuperFrost™ Plus slides.

A Nikon e600 fluorescence microscope was used, and the images were photo documented with a Nikon DXM 1200F color camera and software (Nikon, Tokyo, Japan).

Bacteria were quantified using group-specific C3 probes. The FITC-marked universal probe was used in each hybridization to evaluate the total number of bacteria, and the C5-marked probe with a different probe specificity than C3 was used to determine the spatial relationship of different bacterial groups to each other.

Only signals that hybridized with a specific FISH probe and the universal FISH probe but did not hybridize with specific FISH probes from unrelated bacterial groups were evaluated [13].

Bacterial concentrations of homogeneous populations were enumerated visually in one of the  $10 \times 10$  fields of the ocular raster corresponding to  $10 \mu\text{m} \times 10 \mu\text{m}$  of the section surface at a magnification of  $\times 1000$ . This number was assigned to a concentration of  $10^9$  bacteria/mL, which was the most equivalent to a calculation formula used previously [15].

In case of uneven distribution of bacteria over the microscopic field, the positive signals were enumerated in 10 fields of the ocular raster along the distribution gradient and an average was used after dividing by 10.

### Bacterial groups and FISH probes investigated

A total of 180 bacterial FISH probes available from public resources [10] were applied. Hybridizations were performed with all probes (Table 1 and Table S1), but 31 of these probes were excluded from the analysis (Table S1) because they showed multiple uncharacteristic signals, both in form and distribution. These probes did not cross-react with any of the related bacterial groups, or the seven FISH probes that were identical to the related probes for the same species.

**Table 1**  
Applied FISH probes.

Substantial groups
<b>Essential</b> (N = 3)
<b>Erec482</b> ( <i>Eubacterium rectale</i> , <i>Clostridium coccooides</i> group)
<b>Bac303</b> (most <i>Bacteroidaceae</i> )
<b>Fprau</b> ( <i>Faecalibacterium prausnitzii</i> )
<b>Individual Pioneer</b> (N = 5)
Bif153 Genus <i>Bifidobacterium</i>
Cdif198 <i>Clostridium difficile</i>
Clit135 <i>Clostridium lituseburense</i> group including <i>C. difficile</i>
Ebac1790 <i>Enterobacteriaceae</i>
GAM42a <i>Gammaproteobacteria</i>
<b>Complementary</b> (N = 39)
ACA652/ACA23A <i>Acinetobacter</i>
ACI623 <i>Acidaminococcaceae</i> sp. (not the <i>Selenomonas</i> species)
AKK406 <i>Akkermansia</i>
Ato291 <i>Atopobium</i> cluster
Bcat187 <i>Bifidobacterium. catenulatum</i> group
Bcv13b <i>Burkholderia vietnaminis</i> , <i>Burkholderia cepacia</i>
Bdis656 <i>Bacteroides distasonis</i>
Bifado434 <i>Bifidobacterium. adolescentis</i>
Blon1004 <i>Bifidobacterium. longum</i>
Bputre698 <i>Bacteroides putredinis</i>
Burkho <i>Burkholderia</i> spp.
Ceut705 <i>Coprococcus eutactus</i> , <i>Coprococcus</i> sp.
Chis150 <i>Clostridium histolyticum</i>
Cor653 <i>Coriobacterium</i> group
Cvir1414 <i>Clostridium viride</i> group
DSS658 <i>Desulfobacteraceae</i> and others
Ecy1387 <i>Eubacterium cylindroides</i>
Ehal1469 <i>Eubacterium hallii</i>
HEL274 <i>Helicobacter</i> sp., <i>Wolinella</i> sp.
Lab158 <i>Lactobacillus</i> sp., <i>Enterococcus</i> sp.
Lach571 <i>Lachnospira multipara</i>
LGC354b <i>Firmicutes</i> (Gram-positive bacteria with low G+C content)
MIT447 <i>Streptococcus mitis</i>
Muc1437 <i>Akkermansia muciniphila</i>
Myc657 <i>Mycobacterium</i> subdivision (mycolic acid-containing <i>Actinomycetes</i> )
Pce <i>Burkholderia</i> spp.
Phasco741 <i>Phascolarctobacterium faecium</i>
Pnig657 <i>Prevotella nigrescens</i>
ProCo1264 <i>Ruminococcus productus</i>
Rbro730 <i>Clostridium sporosphaeroides</i> , <i>Ruminococcus bromii</i> , <i>Clostridium leptum</i>
Rfla729 <i>Ruminococcus albus</i>
SFB1 Segmented filamentous bacteria
SNA <i>Sphaerotilus natans</i>
SPH492 <i>Sphingomonas</i> , <i>Erythrobacter</i>
Strc493 most <i>Streptococcus</i> spp.
SUBU1237 <i>Burkholderia</i> spp., <i>Sutterella</i> spp.
TT1372 <i>Treponema</i>
Urobe63a <i>Ruminococcus obeum</i> -like
Ver620 <i>Verrucomicrobium</i>

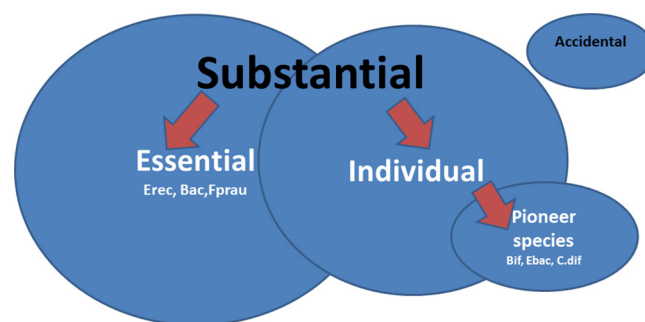
The names of the FISH probes are listed according to abbreviations of the probeBase online resource <http://www.microbial-ecology.net/probebase/default.asp?mode=search>. The Fprau probe is described in [20].

### Statistical analysis

Differences between groups were evaluated using the Mann–Whitney *U* test. Data are presented as means  $\pm$  SD and  $p < 0.05$  was considered to be statistically significant.

### Results

One woman in group A, one women in group A/Sb and two women in group A.Sb discontinued the antibiotic therapy after 3–5 days because of side-effects, in particular, nausea ( $n = 3$ ) accompanied by diarrhea ( $n = 2$ ) and a facial rash ( $n = 1$ ). The remainder of the participants completed the antibiotic therapy and Sb prophylaxis without noteworthy side-effects. Hence, complete data could be obtained from 19, 19 and 18 women in the A, A/Sb, and A.Sb groups, respectively. The number of stool cylinders analyzed was



**Fig. 1.** Composition of colonic microbiota.

188 out of 228 in group A, 170 out of 228 in group A/Sb, and 172 out of 216 in group A.Sb.

### Microbiota in the pre-antibiotic period

#### Occurrence, concentrations and variability

Single bacterial groups could be divided into categories denominated substantial or accidental, depending on their prevalence, abundance and distribution in the stool cylinder. Substantial groups occurred in at least 20% of the population at concentrations of  $> 10^9$  bacteria/mL in at least one of the samples. Accidental bacterial groups occurred in less than 10% of the fecal samples in concentrations never reaching  $10^9$  bacteria/mL and in most cases lower than  $10^7$  bacteria/mL.

Within the substantial groups, bacteria detected with the EREC, Bac, and Fprau FISH probes (mainly *Roseburia*, *Bacteroides*, *Faecalibacterium prausnitzii*) were consistently present in each sample of each person at concentrations between  $8 \times 10^9$  and  $30 \times 10^9$  bacteria/mL. Therefore, the invariability and predominance of these bacterial groups in humans was obviously essential for colonic fermentation, and they were denominated the essential bacteria. However, in earlier publications, we have used the term “habitual” in order to stress the obligate presence of these groups in healthy persons.

All other substantial groups were present in only some patients, and they were denominated individual substantial groups. Since their concentrations could reach  $10^{10}$  bacteria/mL, they added significantly to the colonic microbiome, together comprising approximately 50% of the biomass.

Pioneer species were subgroups of individual substantial bacteria with preferential growth after antibiotic-induced suppression of colonic microbiota, but they will be described later.

The resulting study-related classification of microbial groups is shown in Fig. 1.

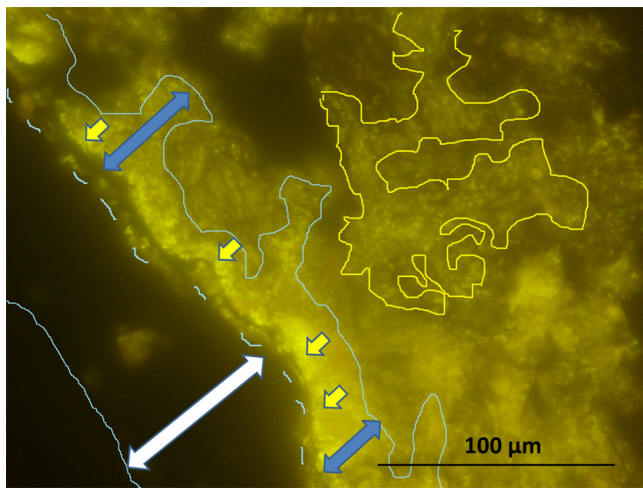
#### Individual microbial profile

The individual substantial bacterial groups were either permanently present or absent in the three monthly collected fecal samples prior to therapy in 93% of the subjects, while in 7% they were transient.

The consecutive presence, absence or transience of colonization by individual substantial bacteria was unique for each person and constructed a characteristic stable individual microbial profile.

#### Anatomy of the colonic bioreactor

The fecal cylinder included a superficial mucus layer, a transitional layer and a fermentation area (Fig. 2). Bacteria were absent or only sporadically present in the 10–100  $\mu$ m broad external mucus layer, and highly concentrated in the fermentation area and transitional mucus.



**Fig. 2.** Spatial setup of the colonic bioreactor: Hybridization with Bac303 (*Bacteroides*) Cy3 (orange fluorescence) probe, patient AG, day –30. The interior of the colonic bioreactor is composed of three zones: a 10–100  $\mu\text{m}$  broad separating mucus layer lacking bacteria (white arrow); a 10–30  $\mu\text{m}$  broad transitional mucus layer homogeneously infiltrated by bacteria of the substantial groups (blue arrow); and a central fermenting area. The central fermenting area merges rich nutrient digestive leftovers and gas vacuoles with fermentative pulp. The mucus of the separating and transitional layer does not include digestive leftovers. Substantial bacterial groups are highly concentrated in the transitional layer. The luminescence of their fluorescence is accentuated on contact with mucus (yellow arrows). In fact, the fermentative pulp (demarcation with a yellow line) represents the offshoots of the transitional layer spreading between digestive leftovers. For background fluorescence, the mucus can be seen as a shadow framing the outer portion of the stool cylinder.

The change from bacteria-free mucus to mucus occupied by bacteria was abrupt, with bacterial concentrations jumping from zero to a maximum without any perceptible gradient. This abrupt change generated a bacterial front that lined the border between separating mucus and the rich bacterial transitional area on the mucosal side of the fecal cylinder. The intensity of fluorescence was accentuated along the line of the bacterial front, demonstrating optimal growth conditions and bacterial metabolism on contact with the mucus. At 10–40  $\mu\text{m}$  behind this front, bacteria were evenly distributed within a homogeneous belt of transitional mucus, which contrasted with the irregularly composed fermentation area.

Fermentation needs both substrates and bacteria that are noticeable ideally as soon as the spatial structure of the stanced fecal cylinders is evaluated. The fermentation area had an appearance of corned meat in which microbial pulp was intermixed with bizarrely formed and structured particles of digestive leftovers and gas vacuoles. Unlike the mucosal side, the change from the homogeneous transitional mucus to the patchy fermentation area was irregular. Transitional mucus contained multiple offshoots, which intruded more or less deep into the interior of the fermentation area. The bacterial gradient extended from the rich bacterial transitional mucus to leftovers of the fermentation area. In fact, the microbial fermentation pulp could be regarded as detached branches of the transitional mucus inseminating the digestive leftovers (Fig. 2).

The distribution of the bacterial groups between the transitional layer and the fermentation area depended on whether they were substantial or accidental. Accidental bacteria avoided the transitional layer, and remained solitary or formed small groups within the stirred fermenting area. No preference for their location to any specific region could be perceived, whereas substantial bacterial groups were located in both the fermentation and transitional areas.

The bacterial front arising from transitional mucus had a different position for the single substantial groups: *Verrucomicrobia* (Muc, Akk, Hel274) were found to be most external, followed by *Enterobacteriaceae* (Ebac, Gam) and then *Roseburia* (EREC), while *Bacteroides* (Bac303) and *Bifidobacteriaceae* (Bif153, Bif164) were most internal toward the center of the fecal cylinder, leading to the impression that *Verrucomicrobia* preferred mucus, whereas *Bifidobacteriaceae* avoided the mucus [15]. However, since none of the substantial groups demonstrated suppression on contact with mucus, and all substantial bacterial groups exhibited preferential growth and accentuation of fluorescence within the transitional area, the correct interpretation should be that substantial bacterial groups differed in their ability to penetrate the mucus, drifted apart and imitated a natural “gel electrophoresis” while occupying their group-specific levels.

#### Antibiotic-induced changes

Antibiotics markedly suppressed most of the substantial bacterial groups (Table S2). The suppression lasted an average of two weeks after discontinuing the antibiotic treatment. Recovery then became perceptible after six weeks and was gradual thereafter. However, the mean concentrations of most substantial bacterial groups were still below the initial values at follow-up, 130–160 days after antibiotic treatment.

The recovery dynamics were different for *Enterobacteriaceae* (Ebac/Gam), *Bifidobacteriaceae* (Bif153) and *Clostridium lituseburense/difficile* (Clit135/Cdif198), since their mean concentrations increased exponentially after discontinuing the antibiotics, and the maximum concentrations were observed two to four weeks post-antibiotic treatment approximately doubling the pre-antibiotic values. The concentrations decreased likewise exponentially as soon as the recovery of other substantial bacterial groups began, starting with week six.

The dynamic of the accidental bacterial groups was opposite to all substantial bacteria. The incidence and concentrations of accidental bacterial groups increased during antibiotic treatment and declined afterwards, but the concentrations remained extremely low and occurrence was restricted to isolated samples.

To make the tendencies more obvious, the concentrations of essential, individual substantial and accidental bacteria were added for each patient and the dynamics of the mean values were analyzed within each group separately for each corresponding microbiota. Furthermore, *Enterobacteriaceae*, *Bifidobacteriaceae* and *Clostridium lituseburense/difficile* were combined and moved to a separate subgroup of pioneer species.

#### Essential microbiota

Essential bacterial groups were predominant in all samples prior to antibiotic treatment but the complete disappearance of the essential bacteria in some samples during antibiotic treatment was especially striking. *Faecalibacterium prausnitzii* was most vulnerable, disappearing in approximately 30% of the samples, while *Bacteroides* and *Roseburia* disappeared in 5–15% of the samples (Groups A and A.Sb). In patients with concomitant *S. boulardii* treatment (A/Sb), the *Bacteroides* and *Roseburia* groups were not affected and *F. prausnitzii* was less affected, being lost in only 16% as compared to 29% in patients without Sb.

The prompt decrease and complete disappearance of the essential groups in samples collected during antibiotic treatment resulted in a global decline of bacterial numbers by approximately one order of magnitude (Fig. 3A, Table 2A).

Despite this comparatively moderate reduction, post-antibiotic recovery was slow and had no resemblance to the exponential growth typical for batch cultures. Two weeks after antibiotic

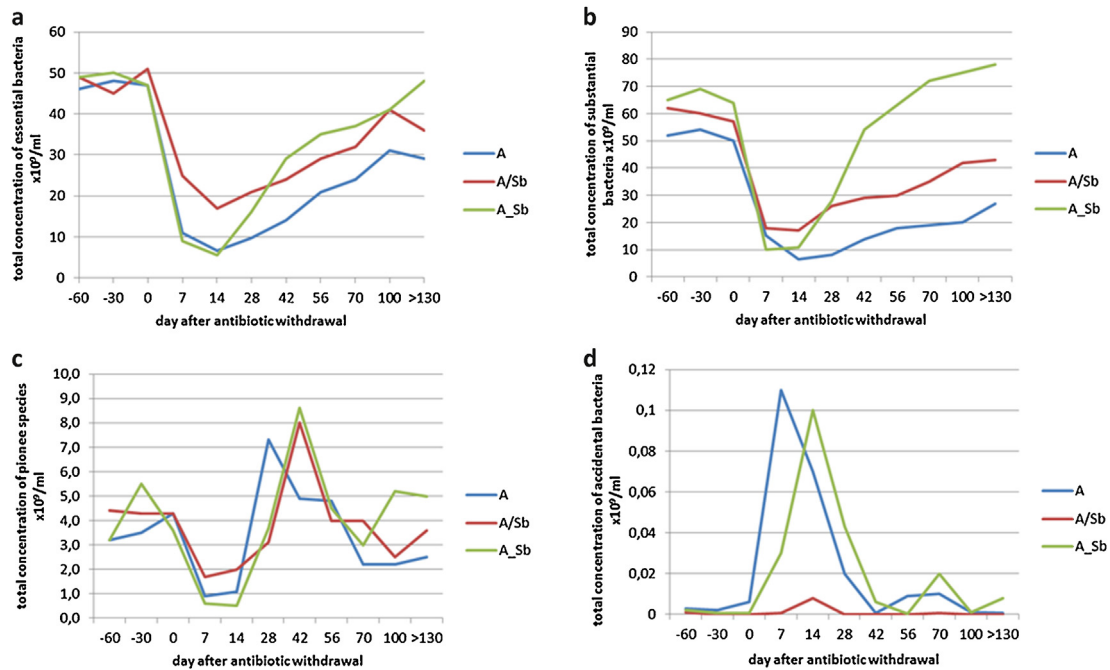


Fig. 3. Shifts in total concentrations of (A) Essential bacterial groups; (B) Individual substantial bacterial groups; (C) Pioneer species; (D) Accidental groups

treatment, the concentrations of the essential bacteria remained similar low as at the time of antibiotic treatment. The first increase in concentrations was only statistically different from values obtained during antibiotic treatment eight weeks post antibiotic treatment. The bacterial concentrations remained significantly below initial values after day 130 in the group receiving antibiotics alone.

*Saccharomyces boulardii* modified the post-antibiotic recovery of the colonic microbiota markedly.

The decrease in bacterial concentrations was significantly less obvious in patients receiving *S. boulardii* together with antibiotics (group A/Sb) compared to the other two groups. The recovery in patients receiving *Sb* after antibiotic treatment (group A\_Sb) was very quick initially, reaching pre-antibiotic concentrations after 4 weeks (Fig. 3A, Table 2A). This was the only group of patients where concentrations of essential bacteria reached pre-antibiotic values at the end of the observation after day 130.

#### Individual substantial microbiota

The total concentration of 39 individual substantial bacterial groups was similar or slightly higher compared to the total concentration of the essential substantial bacteria. Despite the large variation in occurrence and concentrations of single individual bacterial groups, the temporal shift curves in total biomass of the individual substantial bacteria were similar to those observed in the essential microbiota (Fig. 3B, Table 2B).

#### Pioneer species

The decline in concentrations of *Bifidobacteriaceae*, *Enterobacteriaceae* and *C. lituseburensis/difficile* groups was identical to all other substantial groups, however, the recovery curves were different. Within the first two weeks post-antibiotic treatment, the pioneer species doubled their concentrations as compared to pre-antibiotic values (group A).

However, two weeks later, the peak started to decrease as the other substantial bacterial groups started to recover, with the

total concentration reaching the initial values at day 100 (Fig. 3C, Table 2C).

#### Accidental groups

The accidental bacteria increased when substantial groups decreased and vice versa. They appeared more often in concentrations up to  $0.1 \times 10^9$  bacteria/mL under antibiotic treatment and disappeared two to four weeks later (Fig. 3D). Some of the accidental microbial groups, in particular *C. viridae*, *Streptococcus*, *Staphylococcus* and *Bifidobacterium longum*, were detected exclusively in samples during antibiotic treatment, but not before or after the antibiotic treatment. The marked increase of accidental bacteria during antibiotic treatment did not add substantially to the overall colonic biomass because these bacteria occurred in much lower concentrations compared to the substantial bacteria.

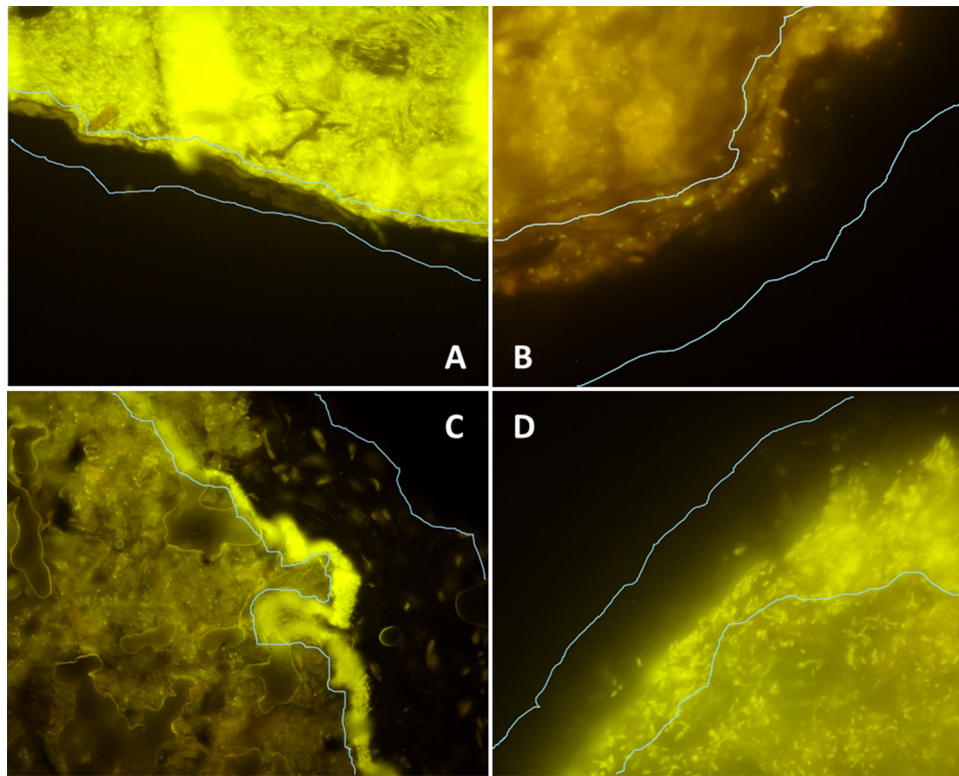
#### Individual microbial profile

Prior to antibiotic therapy the individual substantial bacterial groups were permanently positive or negative in 93–94% of the samples. During antibiotic therapy and shortly thereafter, the occurrence of single bacterial groups in consecutive samples became unpredictable. The stability recovered with increasing duration from the end of antibiotic therapy, however, 4 months after antibiotic therapy the percentage of unstable individual bacterial groups remained at 15% in group A patients, and decreased to the initial value of 7% in both groups of patients receiving *Sb*.

The antibiotics did not only increase the number of volatile groups but also changed the composition of individual microbial profiles. When consecutive pre-antibiotic measurements were compared with consecutive monthly measurements at the end of the study, 43% of individual substantial groups shifted from constantly present to constantly absent, from stable to volatile or vice versa in group A. The individual microbial profiles changed only 12% of the investigated individual bacterial groups in both groups receiving *Sb*.

**Table 2**  
Mean total concentrations  $\times 10^9$  bacteria/mL  $\pm$  SD.

		a -60	b -30	c 0	d 7	e 14	f 28	g 42	h 56	i 70	j 100	k >130	
<i>A Essential bacterial groups</i>													
A	I	46 $\pm$ 4	48 $\pm$ 3.5	47 $\pm$ 9	11 $\pm$ 7	6.7 $\pm$ 3.1	9.7 $\pm$ 6.5	14 $\pm$ 5	21 $\pm$ 8	24 $\pm$ 10	31 $\pm$ 6	29 $\pm$ 9	d to k vs a, b, c $p < 0.01$ – $0.001$ ; e vs f and d vs g = ns; e vs g $p < 0.001$
A/Sb	II	49 $\pm$ 5	45 $\pm$ 8	51 $\pm$ 9.9	25 $\pm$ 10	17 $\pm$ 4	21 $\pm$ 7	24 $\pm$ 7	29 $\pm$ 9	32 $\pm$ 12	41 $\pm$ 14	36 $\pm$ 6	d to I vs a, b, c $p < 0.01$ – $0.001$ ; e vs f $p < 0.001$ ; c vs j, k ns
A.Sb	III	49 $\pm$ 7	49 $\pm$ 8	47 $\pm$ 6	9 $\pm$ 5	5.5 $\pm$ 4	16 $\pm$ 6	29 $\pm$ 8	35 $\pm$ 9	37 $\pm$ 10	41 $\pm$ 10	48 $\pm$ 9	d to h vs a, b, c $p < 0.05$ – $0.001$ ; d, e vs f $p < 0.001$ ; c vs k ns
					II vs I, III $p < 0.001$	II vs I, III $p < 0.001$	I vs II, III $p < 0.05$ – $0.001$	I vs II, III $p < 0.001$	I vs II, III $p < 0.001$	I vs II, III $p < 0.001$	I vs II, III $p < 0.001$	I vs II, III $p < 0.001$	
<i>B Individual substantial bacterial groups</i>													
A	I	52 $\pm$ 24	54 $\pm$ 22	50 $\pm$ 22	15 $\pm$ 14	6.4 $\pm$ 4.7	8.2 $\pm$ 5.5	14 $\pm$ 8.1	18 $\pm$ 8.9	19 $\pm$ 8.2	20 $\pm$ 12	27 $\pm$ 14	d, e to k vs a, b, c $p < 0.001$ ; e vs f and d vs g = ns; e vs g $p < 0.001$
A/Sb	II	62 $\pm$ 26	60 $\pm$ 26	57 $\pm$ 22	18 $\pm$ 12	17 $\pm$ 10	26 $\pm$ 13	29 $\pm$ 12	30 $\pm$ 12	35 $\pm$ 14	42 $\pm$ 16	43 $\pm$ 16	d to j vs a, b, c $p < 0.05$ – $0.001$ ; e vs f $p < 0.02$ ; c vs k ns
A.Sb	III	65 $\pm$ 20	69 $\pm$ 19	64 $\pm$ 16	10 $\pm$ 4.4	11 $\pm$ 6.4	28 $\pm$ 14	54 $\pm$ 17	63 $\pm$ 13	72 $\pm$ 15	75 $\pm$ 13	78 $\pm$ 14	d to f vs a, b, c $p < 0.001$ ; d, e vs f $p < 0.001$ ; abc vs h, i, j, k - ns
		I vs II, III ns	I vs II, III ns	I vs II, III ns	III vs II $p = 0.01$	II vs I, III $p = 0.05$ - $< 0.001$	I vs II, III $p < 0.001$	I vs II, III $p < 0.001$ II vs III $p < 0.001$	I vs II, III $p < 0.001$ II vs III $p < 0.001$	I vs II, III $p = 0.002$ II vs III $p < 0.001$	I vs II, III $p = 0.03$ II vs III $p = 0.002$	d, e to k vs a, b, c $p < 0.001$ ; e vs f and d vs g = ns; e vs g $p < 0.001$	
<i>C Pioneer bacterial groups</i>													
A	I	3.2 $\pm$ 3.4	3.5 $\pm$ 3.2	4.3 $\pm$ 4.6	0.9 $\pm$ 1.8	1.1 $\pm$ 1.3	7.3 $\pm$ 5.9	4.9 $\pm$ 5.3	4.8 $\pm$ 5.3	2.2 $\pm$ 2.5	2.2 $\pm$ 1.9	2.5 $\pm$ 2.7	f vs a, b, c, d, e, g, h, i, j, k $p < 0.05$ – $0.001$
A/Sb	II	4.4 $\pm$ 5.1	4.3 $\pm$ 5.3	4.3 $\pm$ 5.3	1.7 $\pm$ 2.9	2 $\pm$ 4.9	3.1 $\pm$ 5.9	8 $\pm$ 7	4 $\pm$ 4	4 $\pm$ 3.8	2.5 $\pm$ 2.8	3.6 $\pm$ 3.5	g vs a, b, c, d, e, f, h, i, j, k $p < 0.05$ – $0.001$
A.Sb	II	3.2 $\pm$ 3.6	5.5 $\pm$ 3.9	3.6 $\pm$ 2.9	0.6 $\pm$ 1	0.5 $\pm$ 0.5	3.7 $\pm$ 4.4	8.6 $\pm$ 7.5	4.5 $\pm$ 4	3 $\pm$ 1.6	5.2 $\pm$ 3.9	5 $\pm$ 3.7	g vs a, b, c, d, e, f, h, i, j, k $p < 0.05$ – $0.001$
										I vs II, III $p < 0.05$	I vs III $p < 0.05$	I vs III $p = 0.05$	



**Fig. 4.** Shifts in spatial distribution of bacteria following antibiotic treatment and convalescence. Four cylinders from patient NG in group A collected at day –30, 14, 28, 130 (A, B, C, D) hybridized with the Fprau (*Faecalibacterium prausnitzii*) Cy3 (orange fluorescence) probe: (A) Bacteria are highly concentrated and homogeneously distributed over the stool cylinder except in the separating mucus layer (magnification  $\times 100$ ). (B) Bacteria have disappeared from most parts of the fecal cylinder during antibiotic treatment. Single cells can still be seen in the transition area between the mucus layer and the fermentation area (magnification  $\times 1000$ ). (C) The recovery of bacteria after cessation of antibiotic treatment starts in the transitional area, where bacterial concentrations locally exceed the pre-antibiotic values (magnification  $\times 400$ ). (D) With increasing time after antibiotic therapy, bacteria spread over the whole surface of the fecal cylinder, and the concentrations between the transitional and fermentation area become similar (magnification  $\times 1000$ ).

#### Spatial aspects of bacterial distribution during and post-antibiotic treatment

Shifts in the distribution of substantial bacterial groups were principally the same for essential, individual and pioneer groups; however, they were quite different for the accidental groups.

The antibiotics suppressed fluorescence and the concentrations of substantial bacteria mainly in the central area of the fecal cylinder, however, representatives of the same groups could remain in low concentrations within the transitional area, after they completely disappeared in the fermentation area (Fig. 4B–D). Conversely, restitution started first in the transitional area after antibiotic treatment (Fig. 4C).

The concentration of reviving substantial bacterial groups within the transition area often exceeded two- to tenfold the concentration in the same patient prior to antibiotic treatment. Despite this local outburst, the overall concentrations of bacterial groups in the first stool sample after antibiotic treatment were low, since bacterial concentrations in the fermentation area remained low or absent (Fig. 4C). With increasing time from the antibiotic treatment, a wave of bacterial growth spread over the cylinder with bacterial concentrations equalizing between the transitional and fermentation area (decreasing in the transitional and increasing in the fermentation area, Fig. 4D). The total concentrations of bacterial groups in the fecal cylinder increased accordingly.

The increase in local bacterial concentrations in the transitional area and their spread toward the center of the fecal masses were typical also for patients receiving *Sb*.

No gradients in distribution over the fecal cylinder were observed for accidental bacteria at any time.

#### Discussion

In medicine, the colon is associated with multiple diseases, such as carcinoma, adenoma, appendicitis, diverticulitis, peritonitis, fistula, abscesses, inflammatory bowel disease, ileus, megacolon, diarrhea and constipation, among others. The only positive clinical features are convenient stool frequencies and consistencies. Therefore, the question has to be asked as to why evolution would preserve such a risky organ.

In fact, the reason is simple and evident because the colon is a sophisticated bioreactor. The modern industrial bioreactors that produce drugs, foods or clear toxic waste achieve concentrations of approximately  $10^{10}$  bacteria/mL for limited periods of time. The colonic bioreactor is far more advanced and maintains concentrations of approximately  $10^{12}$  bacteria/mL for years and uses more than 5000 different species for fermentation [12]. Obviously, nature discovered and perfected colonic bioreactors long before bio-engineering. The merits are impressive because, in humans, the colon contributes to energy balance via butyrates and short chain fatty acids, and it is important in prevention of multiple metabolic disorders, such as diabetes mellitus type II and obesity, amongst others. In the horse and rabbit, the fermentation products of the colonic microbiota cover up to 80% of the hosts' energy requirements. The colonic bacteria also produce vitamins [7]. Our ancestors lived on unbalanced mono-diets for long periods of time and colonic bio-fermentation would have been a powerful tool to compensate for any deficiencies, and there are many well-known examples from the animal kingdom that demonstrate this.

Furthermore, the colon is one of the central organs for immune competence – a comprehensive library of complex antigens, which

can all be handled without dangerous contact with associated pathogens [8]. However, the colon develops its function through a complex polymicrobial biomass, which the currently available analytical tools have difficulties to monitor. Previous studies were mainly aimed at microbial identification, first by culture, and later by culture-independent methods [4,6,12]. The microbiomes were regarded as independent players, occupying a colonic niche according to their intrinsic properties and thus contributing to health or disease. Nevertheless, this can be regarded as an oversimplification, since there is no precedence in wildlife where bacteria achieve comparable concentrations and complexity as they do in the colon or any other evolutionary comparable organs. All vertebrates have a colon and the colonic microbiome is acquired. However, despite similar environments and feeding under controlled breeding conditions, each species maintains its specific microbial profile. Obviously, the microbes do not drive the colon, but the colon selects, forms, facilitates and protects the bacterial biomass according to its functional goals and environmental availability in a process of mutual coevolution [5].

Our data emphasize this remarkable ability of the colon to maintain species and an individual specific, highly concentrated bacterial mass over a long period of time.

In this study, greater than  $10^{11}$  bacteria/mL were detected when 142 different bacterial groups were simultaneously monitored by FISH. However, the total concentration of colonic bacteria decreased tenfold after two weeks antibiotic treatment with ciprofloxacin and metronidazole. The remaining concentration was, however, higher than  $10^{10}$  bacteria/mL, which is still more than most laboratory bioreactors achieve.

It could be supposed that some of the bacteria within the enormous diversity of the colonic microbiome were antibiotic-resistant, and re-occupied the vacated niches of eliminated competitors keeping the total concentration high; however, there were no indications for such displacement within two weeks of antibiotic therapy. Within the spectrum of applied FISH probes, the residual biomass was composed of preexisting substantial microbial groups, most of which were suppressed and none increased.

The astonishing resilience of the colonic microbiome is inexplicable in terms of microbial composition or bacterial resistance to antibiotics. Therefore, the colon's function has to be included in evaluating the microbiome.

In the present study, the structure of bacterial growth was investigated in relation to the colonic wall in sections of fecal cylinders. Spatially, this bioreactor was composed of three areas: separating mucus, a transitional area, and a fermentation area. The digestive leftovers and bacteria were peristaltically stirred and fermented in the fermentative area. However, the role of mucus and its layers is more complex, since water and electrolyte resorption solidify the mucus. The positive viscosity and negative osmotic gradient are highest close to the colonic wall, making mucus impenetrable for bacteria [14]. The impenetrable mucus layer separates mucosa from the colonic microbiota, which is already important in a normally functioning bioreactor, since the highly diverse colonic biomass invariably includes potentially life threatening pathogens, such as *Bacteroides fragilis*, *Clostridium perfringens*, *Enterobacteriaceae* and others. However, this is even more important in the case of the presence of specific enteric infectious agents, such as *Shigella* and *Yersinia enterocolitica*.

The role of the mucus layer, however, exceeds mechanical separation because it is also decisive in attraction, shelter and facilitation of microorganisms [17]. These latter issues are prerogatives of the transitional mucus layer, since, with increasing distance to the colonic wall, the mucus becomes increasingly degraded, softened and penetrable for bacteria. Substantial bacterial groups occupy specific levels within this 10–30  $\mu\text{m}$  thick band according to their ability to move in a viscous environment.

The mucus, however, remains viscous enough to be moved along by peristalsis, and it remains part of the mucus layer forming the transitional area. The presence of bacteria within the mucus divides the transitional area from the separating mucus lacking bacteria. The homogeneous texture and absence of digestive leftovers marks the border between transitional mucus and the stirred fermentation area on the luminal side.

The distribution of bacteria in transitional mucus in healthy individuals and during antibiotic treatment discloses the crucial role of this anatomic structure in steering the colonic bioreactor.

The transitional mucus layer contained no digestive leftovers. Nevertheless, substantial bacterial groups increased to their maximum concentrations, and luminescence of FISH was highest within the transitional layer, suggesting that bacteria were not passively infiltrating the mucus, but were actively attracted and facilitated by the host. The attraction was not general but was restricted to selected bacterial strains, which were host-species-specific, as in the case of the essential bacterial groups, or person-specific, as in the case of all individual substantial bacterial groups. Accidental bacteria preferred the rich digestive leftover fermentation area, had no commitment to mucus and were unpredictable in consecutive measurements.

The “rooting” of substantial bacterial groups in the “topsoil” of the transitional mucus associated the bacteria of interest with a stable individual microbial profile, and sheltered them from eradication through antibiotics. While antibiotic suppression was most severe in the central portion of the fecal cylinder, the substantial bacterial groups prevailed within the transitional area after they completely disappeared everywhere else in the fecal cylinder. After discontinuing the antibiotic, one would expect rapid growth in the area of maximum availability of nutrients. Accidental bacteria, which were presumably located within the fecal stream, should have maximum advantage. Nevertheless, the opposite was the case, although the occurrence of accidental bacteria increased during antibiotic treatment and then disappeared after discontinuation of the antibiotics. In fact, the middle of the fecal stream was quickly occupied by pioneer species of *Bifidobacteriaceae*, *Enterobacteriaceae* and the *Clostridium difficile* groups. The pioneer bacteria increased exponentially to concentrations significantly exceeding the pre-antibiotic values and declined as soon as the fecal cylinder was occupied by other less quickly recovering substantial bacterial groups. The recovery after antibiotic treatment did not start in the center of the fecal stream but within the transitional area. Concentrations of recovering substantial groups in this area exceeded the pre-antibiotic values, although they remained absent in the fermentation area. The local concentrations in the transitional area declined with the spread of bacteria over the fermentation area, and with bacterial distribution equalizing in the fecal cylinder.

Bacterial concentrations of less than  $10^4$  bacteria/mL cannot be reliably detected by FISH, since the volume of the 4  $\mu\text{m}$  thick and 4 mm  $\times$  4 mm large fecal section is too small. Disappearance of substantial groups during antibiotic treatment in FISH investigations does not automatically imply their eradication throughout the colon. However, individual substantial groups that became undetectable under antibiotic treatment did have considerable difficulties to recover, revealing close interdependence between the stability of the microbial profile and the overall output of the fermentative biomass.

After antibiotic treatment, the diversity of the colonic microbiome and the total number of bacteria was reduced, and some of the individual substantial bacterial groups could not be revived. The diversity increased through acquisition of new individual substantial groups. As a result, 43% of the initial individual microbial profile changed. Under this circumstance, the total output of the colonic bioreactor recovered slowly and the concentrations at the end of the observation period still remained significantly below



pre-antibiotic values reaching approximately 60% of the initial concentrations. Obviously, the re-composition of the individual microbial profile needed time-consuming complex adjustments that led to a reduction of the fermentative performance.

The decrease in the concentrations of the fermenting biomass and inconsistency of the bacterial diversity were effectively averted by *Sb* prophylaxis. A total of 88% of patients receiving *Saccharomyces boulardii* CNCM I-745 quickly restored their initial individual microbial profiles. The total microbial concentrations recovered completely in the two *Sb* groups within 3 months post antibiotic. However, although they were different shortly after antibiotic treatment, both concomitant and subsequent *Sb*-treated groups were similar at the end of the observation period.

Many lines of reasoning could explain the observed protective *Sb* effects. For instance, it is commonplace that most microbial culture media include yeast extract as a component for promoting bacterial growth. However, most intriguing is the recently described utilization of yeast mannan by bacteria through a selfish mechanism [2]. Mankind has applied fermentation processes for thousands of years. *Saccharomyces* is one of the main fermentative agents responsible for bread, beer, wine and vinegar products. Nevertheless, the interactions of yeast and human microbiota are probably more complex than we presently assume and still have to be unraveled.

However, this study has shown that the functional anatomy of the colonic bioreactor provides transparent criteria for the direct assessment of colon-microbiome interactions and the perturbations caused by antibiotics.

#### Conflict of interest

No conflicts of interest.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.syapm.2015.11.002>.

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