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CAN ONE RESTORE THE INTESTINAL MICROBIOTA? THE EXAMPLE OF *S. BOULARDII*

Functional biostructure of colonic microbiota (central fermenting area, germinal stock area and separating mucus layer) in healthy subjects and patients with diarrhea treated with *Saccharomyces boulardii*

Biostructure fonctionnelle du microbiote colique (zone de fermentation centrale, zone de réserve germinale et couche de mucus séparatrice) chez les sujets sains et chez les patients atteints de diarrhée traités par Saccharomyces boulardii

A. Swidsinski ^{a,*}, V. Loening-Baucke ^a, S. Kirsch ^b, Y. Doerffel ^c

^a Laboratory for Molecular Genetics, Polymicrobial Infections and Bacterial Biofilms and Department of Medicine, Section of Gastroenterology, Charité Universitätsmedizin Berlin, CCM, 10117 Berlin, Germany

^b Asklepios Clinic Teupitz, Neurology, Buchholzer Str. 21, 15755 Teupitz, Brandenburg, Germany

^c Outpatient Clinic, Internal Medicine, Luisenstr. 11-13, Charité Universitätsmedizin Berlin, 10117 Berlin, Germany

Statements: The manuscript includes no personal information about any of the investigated patients which would make a patient informed consent necessary. The study was performed according to the ethical rules included in the Declaration of Helsinki. The investigations were approved by the Institutional Review Board of the Charité Universitätsmedizin Berlin.

Abbreviations: FISH - fluorescence in situ hybridization; Cy3, Cy5, FITC, DAPI - different fluorescent dyes corresponding to green, orange, dark red, and blue colours; Alcian, PAS - classical staining methods for histological sections; DSS - dextran sulphate sodium *S. boulardii* - *Saccharomyces boulardii*

Summary The colonic content can be compared to a spatially structured high output bioreactor composed of three functionally different regions: a separating mucus layer, a germinal stock area, and a central fermenting area. The stool mirrors this structure and can be used for diagnosis in health and disease. In a first part, we introduce a nov-

* Corresponding author.

E-mail address: alexander.swidsinski@charite.de (A. Swidsinski)

el method based on fluorescence in situ hybridization (FISH) of sections of punched-out stool cylinders, which allows quantitatively monitor microbiota in the mucus, the germinal stock and the central fermenting areas. In a second part, we demonstrate the practical implementation of this method, describing the biostructure of stool microbiota in healthy subjects and patients with chronic idiopathic diarrhea treated with *Saccharomyces boulardii*. Punched stool cylinders from 20 patients with chronic idiopathic diarrhea and 20 healthy controls were investigated using fluorescence in situ hybridization. Seventy-three bacterial groups were evaluated. Fluctuations in assembly of 11 constitutive bacterial groups were monitored weekly for 3 weeks prior to, 3 weeks during, and 3 weeks after oral *Saccharomyces boulardii* supplementation. Typical findings in healthy subjects were a 5-60 μm mucus separating layer; homogeneous distribution and fluorescence, high concentrations ($>10 \times 10^{10}$ bacterial/mL) of the three habitual bacterial groups: *Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii*; and low concentrations of the occasional bacterial groups. The diarrhea could be described in terms of increased separating effort, purging, decontamination, bacterial substitution. Typical findings in diarrhea were: increased thickness of the protective mucus layer, its incorporation in the stool, absolute reduction in concentrations of the habitual bacterial groups, suppression of bacterial metabolism in the central fermenting area (hybridization silence), stratification of the stool structure by watery ingredients, and substitutive increase in the concentrations of occasional bacterial groups. The microbial and clinical symptoms of diarrhea were reversible with *Saccharomyces boulardii* therapy. The structure-functional analysis of stool microbiota allows to quantitatively monitor colonic malfunction and its response to therapy. *Saccharomyces boulardii* significantly improves the stool biostructure in patients with chronic idiopathic diarrhea and has no influence on the stool microbiota in healthy subjects.

Résumé Le contenu du côlon peut être comparé à un bioréacteur à haut débit doté d'une structure spatiale comportant trois régions fonctionnellement différentes : une couche de mucus séparatrice, une zone germinale de réserve et une zone centrale de fermentation. Les selles reflètent cette structure et peuvent être utilisées comme un outil diagnostique chez le sujet sain ou malade. Dans la première partie, nous introduisons une méthode innovante fondée sur l'étude par hybridation fluorescente in situ (FISH) de coupes de selles prélevées par carottage, ce qui permet une évaluation quantitative du microbiote dans le mucus, dans la zone de réserve germinale et dans la zone centrale de fermentation. Dans une seconde partie, nous démontrons la mise en œuvre pratique de cette méthode en décrivant la biostructure du microbiote fécal chez des sujets sains et chez des patients atteints de diarrhée chronique idiopathique traités par *Saccharomyces boulardii*. Des carottes de selles de 20 patients souffrant de diarrhée chronique idiopathique et de 20 témoins sains ont été étudiées par la méthode de FISH. Soixante-treize groupes bactériens ont été évalués. Les fluctuations dans la combinaison de 11 groupes bactériens constitutifs ont été observées chaque semaine pendant trois semaines avant, trois semaines pendant et trois semaines après une supplémentation orale par *Saccharomyces boulardii*. Les constatations typiques des sujets sains étaient une couche muqueuse séparatrice de 5-60 μm , une distribution et une fluorescence homogènes, de fortes concentrations ($>10 \times 10^{10}$ bactéries/mL) des trois groupes bactériens habituels - *Bacteroides*, *Roseburia* et *Faecalibacterium prausnitzii* - et de faibles concentrations des groupes bactériens occasionnels. La diarrhée a pu être décrite en termes d'augmentation de l'effort de séparation, d'épuration, de décontamination et de substitution bactérienne. Les constatations caractéristiques de la diarrhée étaient une augmentation d'épaisseur de la couche muqueuse protectrice, son incorporation aux selles, une réduction absolue des concentrations des groupes bactériens habituels, une inhibition du métabolisme bactérien dans la zone centrale de fermentation (disparition du signal d'hybridation), une stratification de la structure des selles par des ingrédients aqueux et une augmentation compensatoire des concentrations des groupes bactériens occasionnels. Les symptômes microbiens et cliniques de la diarrhée étaient réversibles après le traitement par *Saccharomyces boulardii*. L'analyse du rapport structure/fonction du microbiote fécal permet de caractériser quantitativement la dysfonction colique et sa réponse au traitement. *Saccharomyces boulardii* améliore significativement la biostructure des fèces au cours de la diarrhée chronique idiopathique et n'a pas d'effet sur le microbiote fécal des sujets sains.

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Principles of structure-functional compartment analysis of colonic microbiota in health and disease and their practical implementations

Introduction

The evaluation of colonic function is usually regarded as unfeasible. Too many features and too many bacterial species have to be estimated in order to draw valid conclusions [1, 2]. Indeed modern culture-independent methods enumerate more than 5000 different bacterial species in the healthy colon [3]. Although only a small part of them can be cultivated, the comprehensive cultural analysis of a single fecal sample would bring any microbiologist to despair. Metagenomics, which intends to establish a catalogue of individual and disease specific bacterial genes that are present in the colon without their cultural identification, raises more and more terabytes on sequence data and is still far from the end of the mission [4]. Facing this complexity, the physicians rely more on tangible criteria such as blood and mucus, form, colour, smell and consistency of stool rather than on analysis of bacterial compositions, gene sequences, metagenomics, metalipomics and proteomics of the colonic contents. However do we really need millions of identified and ordered species, bacterial genes and products for relevant conclusions? And, confronted with masses of incoherent single details, can we conclude anything of value? Yes, we can. We are going to show that the understanding of the colonic microbiota is not so difficult and that their analysis can be performed in a straight forward, quantifiable and reproducible manner.

Premises for the development of the structure-functional analysis of colonic microbiota

We have first to recall that the colon is a bioreactor in which waste products of digestion are degraded and the water and electrolytes are cleaned and recycled. Since the modern molecular-genetic industry broadly uses bioreactors, we know a lot about how they work and about requirements for biofermentation. Bacterial concentrations up to 10^{10} can be raised under specific conditions over short periods of time in specialized facilities producing biologicals and drugs. These concentrations are regarded a huge success. However in the colonic bioreactor, which is created by nature, concentrations of 10^{11-12} bacteria per ml are maintained over years. It is self evident, that such high concentrations can be achieved only under active facilitation of bacterial growth in absence of any suppression. The lack of suppression enables irrelevant or even harmful bacteria to exist site by site with fermentative strains. Indeed many of the species found in the colon are clearly pathogenic: *Clostridium perfringens* causes gas gangrene, *Bacteroides* causes abscesses, *Enterococcus* sp. cause heart valve endocarditis, *Enterobacteriaceae* cause sepsis. All these bacteria can be found in high concentrations in the large intestine, without inducing severe disease. The reason for trouble-free operation of the colonic bioreactor is a mucus barrier which perfectly separates the digestive sewage from the colonic wall (Fig. 1).

The columnar epithelial cells absorb water and solidify mucus to a dense viscous layer covering the mucosa. With the solidification of mucus, high molecular weight mucins progressively interlink to build a biological pore filter. High viscosity of the mucus hinders the bacterial movements, the cross linked mucins hold back bacteria and probably the large molecules of toxins, while filtering water and electrolytes from the sewage of the fermenting compartment [5]. The diffusion of water from the fecal stream thins (dilutes) and matures the mucus on the luminal site. The peristalsis carries away the matured mucus and incorporates it into the fecal mass. The mucus layer must be continuously renewed.

The viscosity of the mucus that is secreted by the goblet cells at the crypt bottom is significantly lower than the viscosity of the dehydrated mucus film, which is attached to the columnar epithelium. The secreted mucus can not merge with the dehydrated mucus because of differences in consistency. Instead it heaves up the semisolid mucus layer and spreads below it. This mode of replacement protects freshly secreted mucus from bacterial penetration until it is in turn solidified by water absorption and can serve as impenetrable cover.

Waves of secretions and solidification lead to step-like interruption of the viscosity gradient within the separating mucus layer and an onion-like structure is built. Since mucus is transparent, the onion like structure of the mucus can normally not be perceived. However, when mucus is softened by the fluids from the luminal side, bacteria enter the mucus and the differences in the layers of the mucus become visible as can be seen in the cross sections of the healthy colon (Fig. 1) or even better on cross section of the colon in IL-10 gene deficient mice (Fig. 2), which we will discuss later.



Figure 1 A mucus layer separates fecal microbiota from the colonic wall in the distal colon of the healthy wild type mice. Bacteria are visualized using a *Bacteroides* specific FISH probe (Cy3, yellow fluorescence x 400). Characteristic for the healthy colon is a homogeneous distribution of the main bacterial groups within feces and a clear separation of bacteria from the colonic wall by a mucus layer.

The structure of the functional compartments of colonic microbiota

Fluorescence in situ hybridization of the whole intestine filled with stool indicates that functionally, the colonic bioreactor can be divided into three compartments:

- a highly viscous separating mucus layer, which completely separates the colonic sewage from the mucosa and is free of bacteria;
- a central fermenting compartment, in which bacteria and fibers are stirred and biodegraded; and
- a bacteria entrapping compartment or germinal stock area, which is located between the separating mucus layer and the central fermenting compartment.

The germinal stock compartment in human is an outer (luminal) portion of the separating mucus layer. The mucus becomes increasingly diluted by luminal fluids and penetrable for bacteria at the border between solid mucus and the central fermenting compartment. The softened mucus however stays for prolonged times attached to the colonic wall. Bacteria enter these soft portions of the mucus in concentrations inverse to the increasing viscosity gradient and become more and more immobilized. Bacteria, trapped within the semi-soft mucus, are protected against purging events and can be used for renewed settings of the bioreactor after occasional cleanouts, periods of fasting or even antibiotic treatment. These bacteria make up the germinal stock of the colonic bioreactor.

In the sections of filled colon or sections of the native feces, the functional compartments of the colonic bioreactor are seen as differently organized areas.

In healthy subjects, the composition of both the germinal stock area and the central fermenting areas can not be visually distinguished from each other (Fig. 1). When the colonic bioreactor properly functions, the density and variety of bacteria in both compartments are similar. Only minor differences in density indicate the location of the germinal stock area on the border between central fermenting area and impenetrable mucus. In disease, however, both compartments behave contrary and disease specific [6].

Features characterizing malfunction of the colonic bioreactor

What happens in the laboratory when the bioreactor stops to work properly? The laboratory staff discharges the bioreactor, decontaminates the contents, and resets the system with fresh stock. Exactly the same events occur when the colonic bioreactor malfunctions: discharge (or diarrhea), decontamination (suppression of bacterial concentrations within the central fermenting area of the colonic bioreactor) and resetting with bacteria from the germinal stock. In mice, with colitis induced by oral application of dextran sulphate sodium (DSS), a massive reduction of bacteria produced by diarrhea is prominent in the central fermenting area (Fig. 3). The thickness of the separating mucus layer is compensatory increased. A massive local immune response leads to migration of leukocytes into the mucus layer. Leukocytes array the outer portions of the mucus at the border between the separating

mucus and the germinal stock area. Despite purging of the central fermenting area and local leukocyte response in the mucus layer, concentrations of bacteria within the germinal stock area are not affected and stay high compared to the central fermenting area (Fig. 3).

The discharge or diarrhea is one of the ways to reduce the bacterial number in the central fermenting area, decontamination is another one. The decontamination is an initial event in IL-10 gene deficient mice, which occurs long before the first signs of cellular inflammation or diarrhea, and is therefore especially evident (Fig. 2). The bacterial concentrations are massively reduced at the central fermenting area of apparently healthy young IL-10 gene deficient mice, obviously due to substances secreted in the small intestine that suppress bacterial growth. The germinal stock compartment is, similar to the DSS treated mouse, not involved.

Principal of the method

Reduction of bacteria within the central fermenting area of the colonic bioreactor, the thickness and permeability of the separating mucus layer for bacteria and the state of the bacterial stock within the germinal compartment are parameters that can be reliably monitored using fluorescence in situ hybridization. Actually, native feces contain representative portions of all three functional compartments. Unfortunately, all previous microbial investigators used either smears or homogenized samples of stool. The homogenization disrupts the structural assembly of feces and leads to inconsistent measurements. Depending on what compartment prevails within the fecal homogenate, the results of the analysis will be contradictory despite of the same starting material.

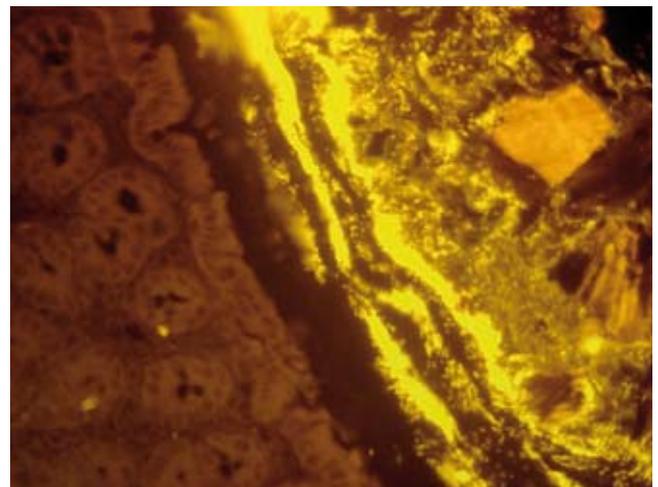


Figure 2 12 week-old IL 10-gene deficient mouse without clinical signs of inflammation, normal weight and formed stool. A massive reduction of bacteria at the central fermenting area can be observed despite lack of symptoms. Bacteria visualized with the universal bacterial probe (Eub338 Cy3, yellow fluorescence, x 1000) are still highly concentrated in the germinal stock area of the colonic bioreactor. An onion-like build up of the separating mucus layer resulting from waves of mucus secretion and solidification is especially well seen in this microphotograph.

Sample Collection and Handling

The 4 to 10 mm long cylinders were punched-out from the stool (Fig. 4) using plastic drinking straws with an inside diameter of 3 mm (Schlecker, Germany). The drinking straws were pre-cut to 4 cm in length and handed out to participants of the study together with 50 ml Falcon tubes filled with 30 ml of Carnoy solution (6/6/1 vol. ethanol/glacial acetic acid/chloroform). Participants were instructed on how to obtain the stool cylinders and given written instructions. The pieces of drinking straw with the stool inside were put into the 50 ml Falcon tube, fixated in Carnoy for 24 hours at room temperature, and then kept refrigerated at 4° C in Carnoy solution until delivered to the laboratory within one to two weeks. In the laboratory, the straws with the enclosed stool cylinder were removed and dipped in black ink to mark the internal portion of the stool cylinder. The stool cylinders were then removed from the straws with a plastic rod, embedded into paraffin using standard techniques, cut longitudinally into 4 µm sections, and placed on SuperFrost plus slides (R. Langenbrinck, Emmendingen, Germany).

Light Microscopy

Alcian blue/Periodic Acid Schiff (PAS) stains were used for evaluation of mucus and leukocytes in the stool specimens and for the orientation within cylinders. The mucus layer at one pole identified the surface of the cylinder, the ink stain on the opposite pole identified the deep or more central portions of the stool.

FISH

A Nikon e600 fluorescence microscope was used. The images were photo documented with a Nikon DXM 1200F color camera and software (Nikon, Tokyo, Japan). Hybridizations were performed in multicolor FISH according to previously described protocols [11,12].

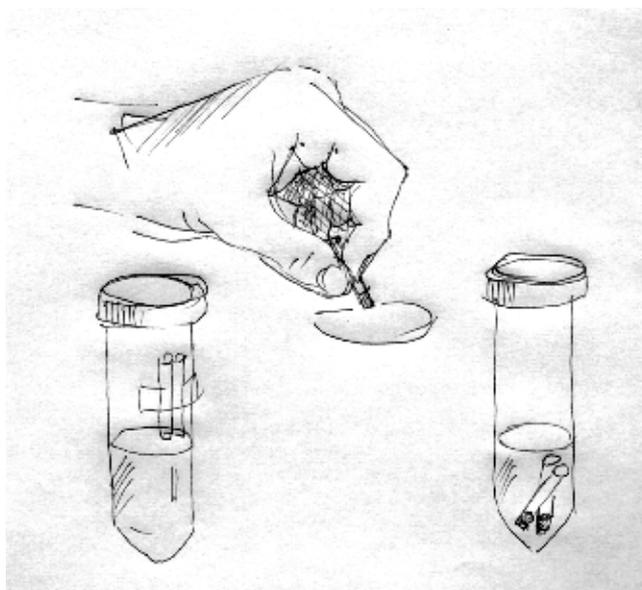


Figure 4 A sample is punched from the stool using pre-cut drinking straws.

Bacteria were quantified using group specific Cy3 probes (orange fluorescence). The FITC marked universal probe (green fluorescence) was used in each hybridization to evaluate all bacteria, Cy5 marked probe (dark red fluorescence) with a difference to Cy3 probe specificity was used to determine the spatial relationship of different bacterial groups to each other. When bacteria occurred in high concentration, they were counted within a 10x10 µm area of the microscopic field. Bacteria with uneven distribution or overall low concentrations were enumerated within larger areas of 100x100 µm, whole microscopic fields, or using the complete surface of the stool cylinder. All enumerations were performed at magnification of 1,000. The conversion of the numbers within defined microscopic areas to concentrations of bacteria per ml was based on the calculation that a 10 µl sample with a cell concentration of 10⁷ cells per ml has 40 cells per average microscopic field. The details of this conversion were previously described [11].

FISH Probes

To select FISH probes, which are most appropriate for longitudinal investigations, the first stool cylinder from each subject participating in this study (N=40) was hybridized with 73 different probes, which were developed for identification of intestinal bacteria, medically relevant isolates and waste water microbial communities (see Table 1) and with the universal Eub 338 probe (Table 1). Fprau and Muc1437 probes are described in the reference [13,14]. The names of all other FISH probes are listed according to abbreviations of probeBase online resource for rRNA targeted oligonucleotide probes (<http://www.microbial-ecology.net/probebase/credits.asp>) [15]. Probes in the table are ordered according to their contribution to the biomass, as explained in the result section.

Statistics

All statistical analyses were performed using the statistical software package SPSS v15.0 (Chicago, Illinois).

Results

Contribution of single bacterial groups to the fecal biomass

Out of 73 investigated bacterial FISH probes only 3 bacterial groups represented by Bac (*Bacteroides* group), Erec (*Roseburia/Clostridium* group XIVa group) and Fprau probes (*Faecalibacterium prausnitzii* group) were detected in each of the samples from each of the healthy and diseased persons (Table 1). In healthy persons these groups of bacteria composed each 15 to 50% of the microbiota and together 70-90% of all fecal bacteria. Because of the presence of these bacterial groups in all stool cylinders in constituting quantities, we termed them habitually constitutive or **habitual** bacteria.

All other investigated bacterial groups were found only in some samples from the same person or they were absent in all samples. The lack of detection of these bacteria within a section of the stool cylinder, does not mean that they are completely absent in feces. However, their quantities in single samples were too low to be detected by FISH.

We developed a novel method in order to overcome the sampling error and to add a spatial resolution to the investigation of fecal microbiota. In analogy to core boring used for investigation of geologic formations, the spatial structure of colonic microbiota was investigated on sections of punched-out stool cylinders, which were fixated and embedded in paraffin. The stool cylinders were collected by patients using precut drinking straw and ready to use 50 ml vials filled with Carnoy solution. The composition and distribution of bacteria within the stool cylinder were analyzed with the use of fluorescence in situ hybridization. We had previously investigated horizontally stool samples from patients with different gastrointestinal diseases and had shown the practical reliability of structure-functional analysis of colonic microbiota for diagnosis of Crohn's disease and ulcerative colitis [7]. It was challenging to prove whether the structure-functional compartment analysis of colonic microbiota is applicable for longitudinal evaluation of symptoms and therapy effects. Of all intestinal disorders, the analysis of diarrheal stool is especially tricky and difficult to perform. To evaluate the feasibility of our method in patients with diarrhea, we chose patients with chronic idiopathic diarrhea treated with the probiotic *Saccharomyces boulandii*.

Changes in the biostructure of fecal microbiota in healthy controls and patients with chronic idiopathic diarrhea treated with *Saccharomyces boulandii*

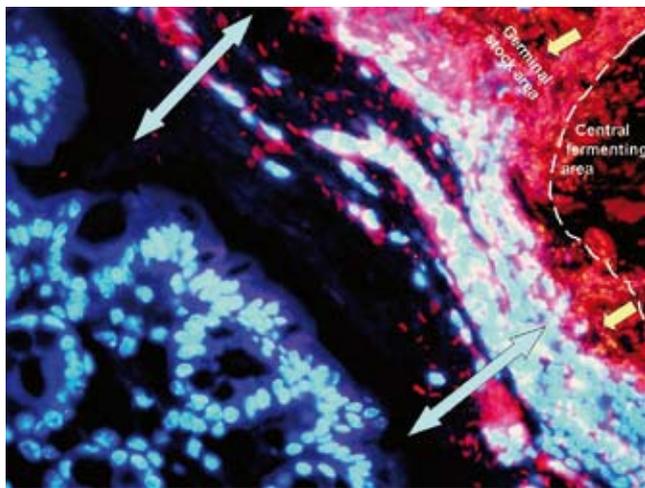


Figure 3 Distal colon of the wild type mouse with colitis induced by oral gavage of water containing DSS. Multicolor FISH using *Bacteroides* (Cy3 probe, yellow fluorescence), *Eubacterium rectale* (Erec Cy5, red fluorescence) and DAPI stain (blue fluorescence, visualizing all DNA structures). With DAPI stain, large nuclei of epithelial cells and leukocytes are well seen. Leukocytes migrate in the mucus and array its outer regions on the border between separating mucus and intestinal contents. The thickness of mucus layer is compensatory increased (double headed arrows). Bacteria are massively reduced in the luminal site of the colon, but stay relatively high at the border between mucus and lumen (yellow arrows), despite a massive leukocyte response (magnification x 1000).

Introduction

Clinically, diarrhea is defined in terms of stool frequency, consistency, volume or weight, the assessment of which affords the cooperation of the patients in keeping a stool record and collecting feces. The data on stool frequency are highly subjective (the difference between drive, tenesm and defecation are vague); the collection of feces over extended periods of time, which is necessary for assessment of volume and weight, is odious and of low informative value. At present, there is no commercial laboratory parameter available that allows the quantification and monitoring of diarrhea and its resolution. While acute diarrhea is in most cases self-limiting, chronic idiopathic diarrhea can persist over months and even years leading to repeated visits to generalists and gastroenterologists. The patients with chronic idiopathic diarrhea report a long list of medications, which were taken previously with no or uncertain response [8]. The symptoms and the patient's perception of the disease are largely fluctuating, which makes the evaluation of drug efficiency based on patients complaints uncertain. In the present study, we longitudinally monitored the biostructure of stool microbiota in healthy controls and patients with chronic idiopathic diarrhea treated with *Saccharomyces boulandii* [9].

We chose *Saccharomyces boulandii* as a tool to challenge the colonic microbiota for the following reasons: *Saccharomyces boulandii* is a yeast and can therefore not interfere with the detection of bacterial species via FISH. Different to antibiotics and Loperamid, it also does not interfere with the mucus layer or with the composition of microbiota. Furthermore of all known probiotics, it is the only drug with proven clinical efficacy in acute diarrhea [9].

Methods

Patients

Twenty patients with chronic idiopathic diarrhea with a duration of at least 12 months and a weekly average of ≥ 4 stools/d (11 males, 9 females, mean age 48.2 years); who had a complete gastroenterological diagnostic investigation, including colonoscopy, gastroscopy, ultrasound and laboratory tests, were enrolled. Patients were considered to have chronic idiopathic diarrhea if they had more than four daily bowel movements for more than 12 months, no systematic illness and no identifiable cause of diarrhea [8, 10].

The healthy controls consisted of laboratory and medical staff and their relatives (8 males, 12 females, mean age 41.2 years), who had no intestinal complaints or known disease.

Study Design

In each group three weeks of surveillance was followed by three weeks of oral supplementation with the yeast probiotic *Saccharomyces boulandii* (250 mg twice a day, Perenterol®, UCB GmbH, 50170 Kerpen, Germany). The analysis of the stool biostructure was also continued over a period of three weeks after the end of the probiotic supplementation. Stool samples were collected weekly for a total duration of nine weeks.

Table 1 FISH probes (ordered according to highest occurrence and concentrations of bacteria in stool)

EUB338 (most Bacteria)

Habitually constitutive bacteria

Erec482 (*Clostridium* group XIVa/*Roseburia* group)
 Bac303 (most *Bacteroidaceae*)
 Fprau (*Faecalibacterium prausnitzii*)

Occasionally constitutive bacteria

Muc1437 (*Akkermansia muciniphila*)
 Ebac1790 (*Enterobacteriaceae*)
 Bif164 (*Bifidobacteriaceae*)
 Ato291 (*Atopobium* cluster)
 Ecy1387 (*Eubacterium cylindroides*)
 Ehal1469 (*Eubacterium hallii*)
 Chis150 (*Clostridium histolyticum*)
 Clit135 (*Clostridium lituseburense* group)

Marginally constitutive bacteria**(For uncertain detection alphabetically ordered)**

ACA652/ACA23A (*Acinetobacter*)
 ACAC (*Actinobacillus actinomycetemcomitans*)
 AERO1244 (*Aeromonadaceae*)
 Alc-476 (*Alcaligenes faecalis*)
 ARC1430 (*Arcobacter*)
 Bcv13b (*Burkholderia vietnamensis*)
 CLOBU1022 (*Clostridium butyricum*)
 Csac67 (*Clostridium* sp.)
 CST440 (*Clostridium stercorearium*)
 DSS658 (*Desulfobacteriaceae*)
 DSV687 (*Desulfovibrionales*)
 E.bar1237 (*Eubacterium barkeri*)
 E.bif462 (*Eubacterium bifforme*)
 E.con1122 (*Eubacterium contortum*)
 E.cyl461 (*Eubacterium cylindroides*)
 E.cyl466 (*Eubacterium cylindroides*)
 E.dol183 (*Eubacterium dolichum*)
 E.had579 (*Eubacterium hadrum*)
 E.len194 (*Eubacterium lentum*)
 E.lim1433 (*Eubacterium limosum*)
 E.mon84 (*Eubacterium moniliforme*)
 E.ven66 (*Eubacterium ventriosum*)
 ENC (*Enterococcus*)

Marginally constitutive bacteria**(For uncertain detection alphabetically ordered)**

FUSO (*Fusobacterium* sp.)
 Fnec (*Fusobacterium necrophorum*)
 Fnuc (*Fusobacterium nucleatum*)
 Hpy-1 (*Helicobacter pylori*)
 Lab158 (*Lactobacillus*)
 Lach571 (*Lachnospira multipara*)
 Myc657 (*Mycobacterium*)
 Pae997 (*Pseudomonas* spp.)
 Phasco741 (*Phascolarctobacterium faecium*)
 PBR2 (*Bifidobacterium breve*)
 Rbro730 (*Clostridium sporosphaeroides*, *Ruminococcus bromii*, *Clostridium leptum*)
 Pce (*Burkholderia* spp.)
 Pden654 (*Prevotella denticola*)
 Pint649 (*Prevotella intermedia*)
 Pnig657 (*Prevotella nigrescens*)
 POGI (*Porphyromonas gingivalis*)
 Ppu (*Pseudomonas* spp.)
 Ppu56a (*Pseudomonas putida*, *P. mendocina*)
 Ppu646 (*Pseudomonas* spp.)
 PRIN (*Prevotella intermedia*)
 ProCo1264 (*Ruminococcus productus*)
 Rfla729 (*Ruminococcus albus*)
 Saga (*Streptococcus agalactiae*)
 Sau (*Staphylococcus aureus*)
 Ser (*Brachyspira*)
 Spn (*Streptococcus pneumoniae*)
 Spy (*Streptococcus pyogenes*)
 Stemal (*Stenotrophomonas maltophilia*)
 SRB385Db (*Desulfobacteriales*)
 Str (*Streptococcus* spp.)
 Strc493 (most *Streptococcus* spp.)
 SUBU1237 (*Burkholderia* spp.)
 STEBA1426 (some members of the *Sterolibacterium* lineage)
 Sval428 (some *Desulfobulbaceae*)
 Urobe63a/Urobe63b (*Ruminococcus obeum*-like)
 Veil223 (*Veillonella dispar*)
 VEPA (*Veillonella parvula*)
 VIB572a (Genus *Vibrio*)

We called these bacterial groups occasional bacteria. The concentrations of occasional bacteria could vary largely and in most cases they were clearly below that of the habitual bacterial groups and reached seldom the threshold of 10%. Eight of the 73 tested bacterial groups reached concentrations of more than 1% of the biomass in at least 5% of the investigated persons, (constitutional for the composition of the fecal mass). All other bacterial groups were either undetectable at the resolution of FISH or contributed far less than 1% of the bacterial mass (marginal for the composition of the fecal masses). The first stool cylinder of each person was investigated using all 73 FISH probes. For longitudinal investigations only FISH probes representing habitual and occasional bacterial groups were used (Table 1).

Structure-functional compartment analysis of colonic microbiota in healthy patients*Habitual bacterial groups*

Habitual bacteria were nearly evenly distributed within the stool cylinder in healthy controls, building a homogeneous texture both in the germinal stock and central fermenting areas. The fluorescence of habitual bacteria was brilliant over the entire surface of the stool cylinder, which confirmed high intensity and output of the biodegradation processes (Fig. 5). The concentrations of *Roseburia* (Erec) were usually higher than that of *Bacteroides* (Bac), and concentrations of Bac were higher than those of *Faecalibacterium prausnitzii* (Fprau), but this rule could deviate in individuals (Table 2).

Occasional bacterial groups

Spatially, occasional bacteria were either diffusely distributed (*Atopobium*, *Bifidobacterium*) or focally condensed (Fig. 6). The differences in concentrations of the focally condensed bacterial groups ranged from 10^{10} bacteria/mL to below detectable within the same sample or between different samples of the same patient. Despite these irregularities, the pattern in occurrence and spatial distribution of occasional bacterial groups were characteristic for each healthy person. The intra-individual variation between single stool samples over the duration of the study was low, and it was often possible to allocate single individuals based on the visual imprint of their microbiota.

Bacteria within mucus layer

The separating mucus layer was in the outside (mucosa facing) portions practically bacteria free. Only a very small fraction of bacteria represented by all groups with an exception of *Bifidobacteria* entered mucus at the fecal side of the mucus layer. While entering mucus, bacteria did not change either their form or their fluorescence, indicating a lack of secreted soluble suppressors within mucus. Bacterial groups could be divided into fecomucous, mucotrop, or mucophob with regard to the mucus layer and the stool surface.

All habitual and most of the occasional bacteria were fecomucous: *Roseburia* (Erec), *Bacteroides* (Bac), *Faecalibacterium prausnitzii* (Ffrau), *Eubacterium cylindroides* (Ecy), *Clostridium histolyticum* (Chis) and *Clostridium lituseburense* (Clit). Their concentrations were highest in lumen, but they could also enter the separating mucus layer in low concentrations. The behavior of mucophob and mucotrop bacteria was apparently different, indicating their peculiar relationship to the mucus layer.

Mucotrop bacteria were represented by two groups: *Enterobacteriaceae* (Ebac) and *Akkermansia muciniphila* (Muc1437). The mucotrop bacteria were located mainly on the interface between mucus and stool and were either completely absent in the central fermenting area or were at least present in markedly lower concentrations (Fig. 7). To correctly quantify the mucotrop bacteria, the numbers and occurrence of Muc1437 and Ebac groups were separately enumerated for the mucus/stool transition zone (mucotrop Ebac, mucotrop Muc1437) and for the stool regions at least 100 μm below the stool surface (fecal Ebac, fecal Muc1437). The diffusely distributed *Akkermansia muciniphila* (Muc 1437) in the stool was always an extension of the more dominant mucotrop population. The diffusely distributed *Enterobacteriaceae* (Ebac) in the stool occurred independent from mucotrop *Enterobacteriaceae*, indicating that the mucotrop and fecal *Enterobacteriaceae* are represented by phenotypically different bacterial groups.

Bacteria, which hybridized with the Bif (*Bifidobacterium*) probe were mucophob, they avoided mucus adjacent regions following fecomucous or mucophob bacteria at a distance of 2-25 μm .

Changes of colonic microbiota in healthy controls

Saccharomyces boulardii supplementation had no statistically relevant influence on composition, occurrence or concentrations of the microbiota in healthy controls.

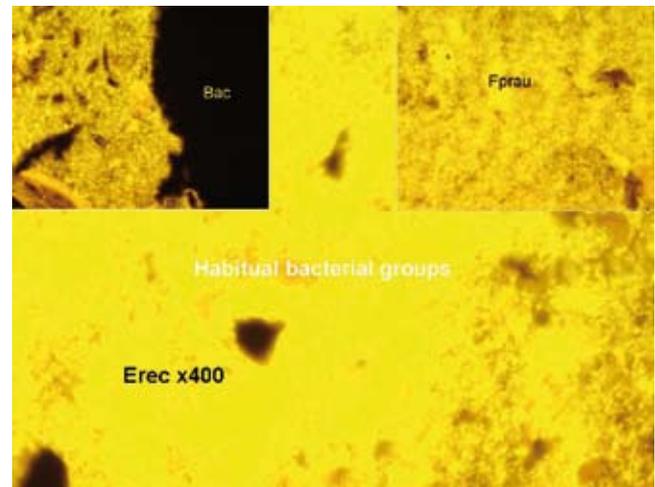


Figure 5 Habitual bacterial groups are evenly distributed throughout the stool cylinder. Hybridization with Cy3 stained probes for habitual bacterial groups x 400.

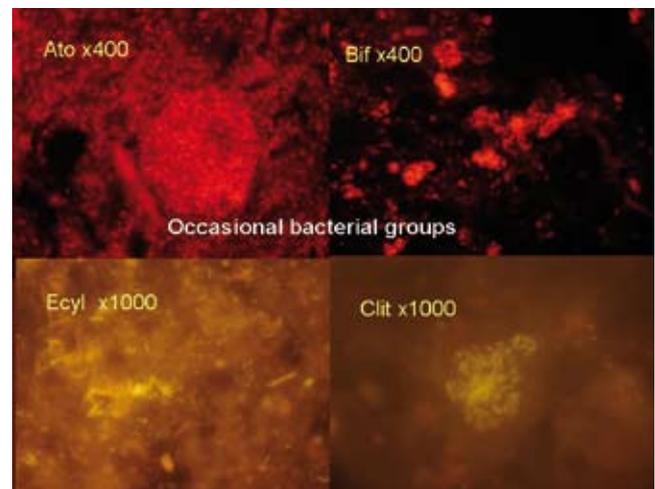


Figure 6 Examples of diffuse and focally condensed occasional bacterial groups.

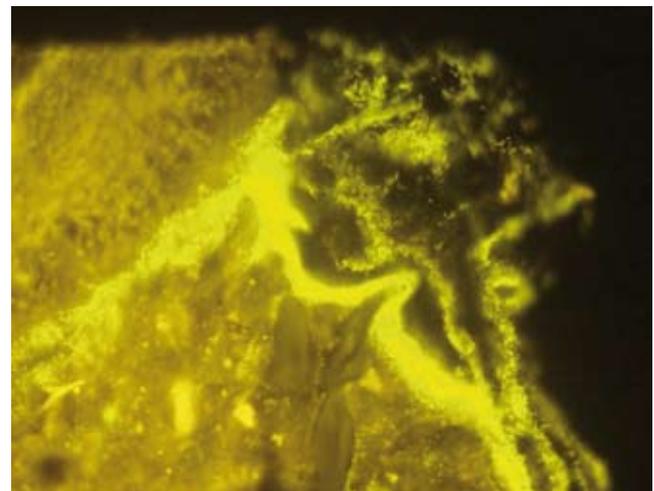


Figure 7 An example of mucotrop bacteria represented by *Akkermansia muciniphila*. Mucotrop bacteria are mainly located on the border between mucus and germinal stock area x 400.

Table 2 Concentrations of single bacterial groups $\times 10^{10}$ per mL over the duration of study.

	Healthy controls*	Patients with diarrhea			
	A	B Previous to treatment	C <i>Saccharomyces boulardii</i> treatment	D After <i>Saccharomyces boulardii</i>	P
Bacterial groups	Mean \pm SD bacterial concentrations				
Habitually constitutive bacteria					
Erec	24.2 \pm 6.1	11.6 \pm 5.0	14.9 \pm 8.3	14.5 \pm 8.9	A/C<0.001 B/C=0.009
Bac	19.4 \pm 5.0	8.8 \pm 4.4	12.6 \pm 6.6	9.2 \pm 5.7	A/C<0.001 B/C=0.014
Fprau	14.9 \pm 3.4	9.4 \pm 4.0	10.8 \pm 4.1	10.1 \pm 4.3	A/C<0.05 B/C ns
Occasionally constitutive bacteria					
Bif	0.58 \pm 1.25	2.09 \pm 2.08	1.46 \pm 1.99	1.71 \pm 2.01	A/C<0.001 B/C ns
Ebac	0.057 \pm 0.21	1.58 \pm 3.22	0.84 \pm 2.29	1.61 \pm 3.42	A/C<0.001 B/C ns
Ato	0.71 \pm 1.04	0.95 \pm 1.06	0.97 \pm 0.98	0.87 \pm 1.74	Ns
EcyL	0.54 \pm 0.82	0.92 \pm 1.23	0.71 \pm 0.91	0.54 \pm 1.07	0.008 //ns
Muc	0.14 \pm 0.73	1.41 \pm 2.68	0.82 \pm 1.79	1.29 \pm 2.54	A/C<0.001 B/C ns
Ehal	0.17 \pm 0.36	0.30 \pm 0.59	0.25 \pm 0.56	0.22 \pm 0.42	A/C=0.04 B/C ns
Chis	0.052 \pm 0.158	0.14 \pm 0.15	0.13 \pm 0.47	0.13 \pm 0.38	A/C=0.017 B/C ns
Clit	0.087 \pm 0.24	0.22 \pm 0.48	0.16 \pm 0.38	0.15 \pm 0.40	A/C=0.004 B/C ns
Occasionally constitutive mucotrop bacteria					
Ebac muc	0.87 \pm 2.58	3.92 \pm 6.45	4.05 \pm 6.42	4.11 \pm 7.2	A/C<0.001 B/C ns
Muc muc	3.49 \pm 7.71	14.82 \pm 17.86	6.58 \pm 10.30	10.65 \pm 18.02	A/C<0.001 B/C=0.002

*Only the pre-treatment results in healthy subjects are listed because the data were not different during and after treatment with *S. boulardii*.

Biostructure of stool microbiota in patients with chronic idiopathic diarrhea and *Saccharomyces boulardii* induced changes

The general classification of stool microbiota as: habitual, occasional; locally, and diffuse distributed; fecomucous, mucophob, mucotrop bacterial groups was also true for the patients with diarrhea. The spatial characteristics in distribution of single bacterial groups in healthy subjects and patients with diarrhea were however markedly different (Table 2).

Reduction in concentrations of habitual bacteria

Habitual bacterial groups remained most predominant in diarrhea, however in each patient at least the threefold reduction in concentrations of habitual bacterial groups was observed when compared to healthy controls (Table 2). This rule had no exceptions. The differences between healthy subjects and patients with diarrhea were highly significant, $P<0.001$.

Substitutive increase in concentrations of occasional bacteria

The concentrations of occasional bacteria were either unchanged (*Atopobium*) or increased (all other bacterial groups) in patients with diarrhea as compared to healthy controls (Table 2). Especially remarkable was a more frequent occurrence and an increase in concentration of the mucotrop bacterial groups both in the stool/mucus transition zone and in the stool core ($P<0.001$), probably due to the increased amounts of secreted mucus. The occurrence

of mucotrop *Akkermansia muciniphila* reached 80% in patients with diarrhea compared to 35% in healthy controls. *Akkermansia muciniphila* was diffusely distributed through the stool in 45% of the patients with diarrhea compared to 15% in healthy controls.

The difference between patients with diarrhea and healthy controls was less extensive but also statistically significant for *Bifidobacterium*, *Eubacterium cylindroides*, *Clostridium histolyticum* and *Clostridium lituseburense*.

The increase of occasional bacterial groups did not substitute for the extensive reduction of the habitual bacterial groups. Taken in absolute numbers, the cumulative increase of all occasional bacterial groups in patients with diarrhea did in no way equalize the losses of the habitual bacterial groups (Table 2).

Stratification of the stool

A feature which was absent in healthy controls but obligate for patients with diarrhea was the interruption of the stool by multiple striae that were arranged in parallel. The striae were usually 5-20 μ m thin, not apparent in alcian stain and most prominent at the central part of the stool cylinder, indicating that the origin of striae was obviously not the mucus, but watery secretions from the small intestine. The homogeneous texture of the habitual bacteria (Fig. 3), which was typical for healthy controls, was interrupted by closely following waves of substance omitting bacteria and leading to characteristic microscopic appearance of "the ruffled by wind water surface" (Fig. 8).

Suppression of fluorescence at the center of stool (hybridization silence)

The habitual bacterial groups were homogeneously distributed all over the surface of the stool cylinder in healthy controls. Although fluctuation in the hybridization intensity could be observed in 25% of healthy controls, these fluctuations did not exceed 50%. The concentration and fluorescence intensity were similar in the central regions and in regions next to mucus in the majority of healthy controls. The situation was quite different in patients with diarrhea. The most prominent feature observed in patients with diarrhea was a suppression of bacterial fluorescence in

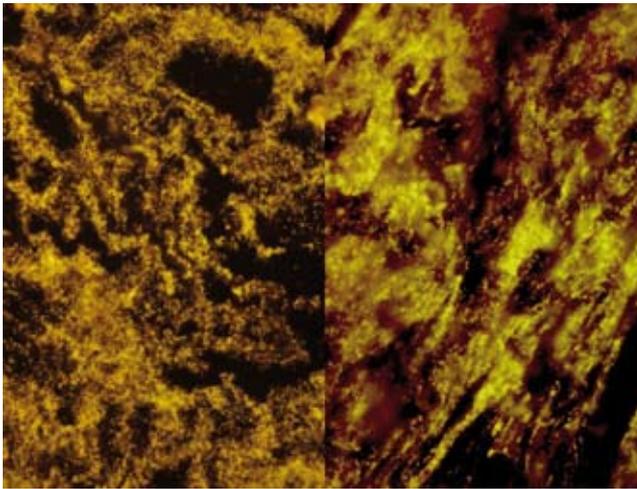


Figure 8 Striae in untreated patients with chronic idiopathic diarrhea. Striae are different to mucus septa, not a result of mucus incorporation in the feces, but a dilution of fecal contents by watery ingredients. Hybridization with the Eub338 probe universal for all bacteria, orange fluorescence, magnification x 400.

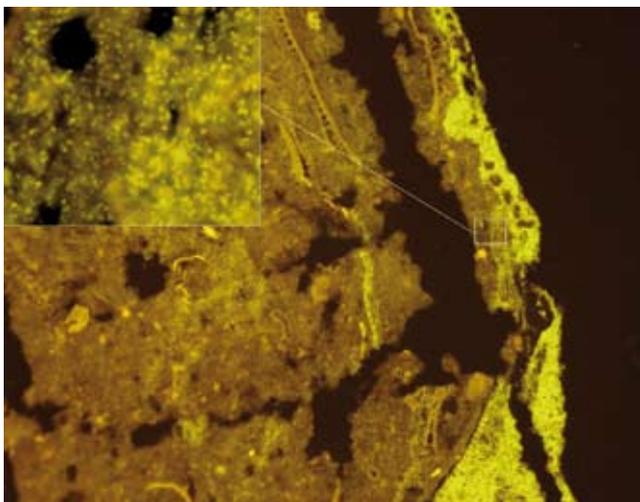


Figure 9 Relative hybridization silence of the *Bacteroides* group in a patient with diarrhea, Bac Cy3 probe, x100. The insertion (x1000) demonstrates that the difference between regions is not only due to differences in bacterial concentrations, but mainly due to suppression of bacterial fluorescence.

the central fermenting area of the colonic bioreactor. We called the phenomenon hybridization silence, since the loss of the fluorescence in the transition zone from excellent to poor fluorescence was gradual, while the number of bacteria remained similar (Fig. 9). Sometimes the suppression was so heavy and extended, that it involved nearly the whole stool cylinder with an exception of a narrow strip of bacteria in the germinal stock area (Fig. 10). There were no indices that suppressive substances were secreted by the mucosa in both patients with diarrhea and healthy controls. In contrast, bacteria located at the border between separating mucus layer and central fermenting area (although massively reduced when compared to healthy controls) had still the highest concentrations and fluorescence within the same stool cylinder.

The quantification of the hybridization silence was expressed as a percent of the stool cylinder surface in which 10% (partial hybridization silence) or less than 0.1% (absolute hybridization silence) of bacteria could be clearly identified. The concentrations of bacteria in the germinal stock area were used as reference for assessment of the hybridization silence. While some of the bacterial groups were heavily affected by hybridization silence, others showed excellent fluorescence all over the cylinder. Commonly the fluorescence of *Bacteroides* was more suppressed than that of *Eubacterium rectale*, and *Eubacterium rectale* more than that of *Faecalibacterium prausnitzii* (Table 3). The hybridization silence was lowest in the *Atopobium* and *Bifidobacterium* groups (not shown). The extent of the hybridization silence for the same bacterial group varied from 2% to 98% between successive samples of the same patient, indicating a highly dynamic state of this phenomenon. Notwithstanding this variability, the involvement of single bacterial groups in hybridization silence was patient specific. In all stool samples from the same patient, one of the habitual bacterial groups was suppressed more than the other, leaving some bacterial groups completely unaffected.

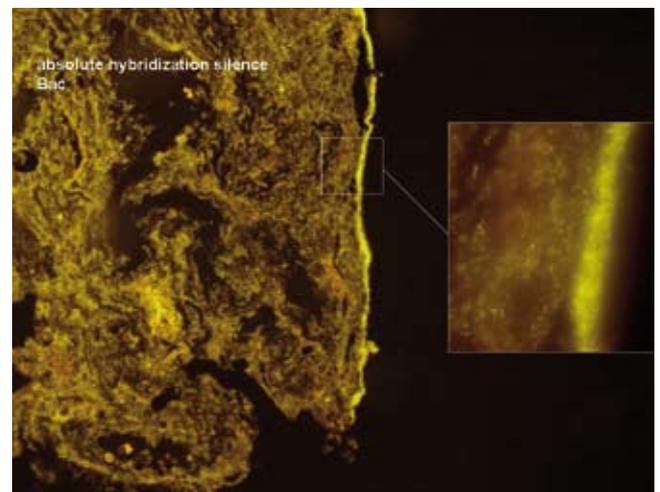


Figure 10 Absolute hybridization silence of the *Bacteroides* group, with bacteria nearly completely extinguished at the central fermenting area of the colonic bioreactor with exception of a small strip of the germinal stock area located next to the mucus layer (x100, insertion x1000).

Table 3 Mean stool frequency/week, hybridization silence, thickness of the mucus layer and striae of the stool cylinder in healthy controls and patients with chronic idiopathic diarrhea.

	Healthy controls	Diarrhea patients			
	A	B	C	D	P
		Previous to <i>S.b.</i> treatment	<i>Saccharomyces boulardii</i>	After <i>Saccharomyces boulardii</i>	
Number of stools/week (mean±SD)		39±16	26±19	29±23	B/C<0.001
Absolute hybridization silence					
Erec	0	35±40	9.6±20.3	21.1±30	B/C=0.001
Bac	0	44±46	14±21	28±32	B/C=0.001
Fprau	0	6±9.4	4±10	9±14	Ns
Presence of a mucus layer	56%	35%	47%	48%	
Thickness of the mucus layer (mean±SD in µm)	35.6±31.7	265±212	96±95	186±209	A/B<0.001 B/C=0.003
Percent of surface covered by striae	0	75.2±30.3	39.7±42.2	50.2±43	A/B<0.001/ B/C<0.001

Alcian stain of the stool cylinder

Separating mucus layer in healthy controls

The surface of the stool cylinders was regularly covered with an up to 36±32µm thick mucus layer in 56% of the stool samples from healthy controls (Table 3). Mucus free of bacteria could also be observed as mucus septa enclosed in stool (Fig. 11), however, the occurrence of septa and their thickness in healthy controls was low.

The separating mucus layer in patients with chronic idiopathic diarrhea

The frequency of finding a mucus cover was lower in patients with diarrhea than in healthy controls (35% versus 56%; $P<0.059$), corresponding to the difficulties collecting an intact cylinder from unformed stool. In samples where the mucus layer stayed preserved, the median thickness of the mucus layer was massively increased in patients with diarrhea compared to healthy controls (176±231 µm versus 36±35 µm; $P<0.001$), indicating the growing separation efforts (Table 3). Samples from patients with chronic diarrhea which had no separating mucus layer had often mucus in form of thick septa enclosed in the fecal masses as shown in Fig. 12. The thickness of the septa in patients with chronic idiopathic diarrhea was increased comparable to the thickness of the mucus cover.

Leukocytes within mucus

No leukocytes were detected within mucus either in healthy controls or patients with diarrhea indicating absence of a local cellular inflammatory response.

Therapeutic effects of *Saccharomyces boulardii*

Clinical effects

S. boulardii treatment, during week 4 to week 6, improved the clinical symptoms in fourteen patients (70%) in regards to the mean number of stools. Stool number decreased

significantly beginning in the first week of intervention (Friedman $\chi^2=72.66$; $p<0.001$) (Fig. 13). The improvement was complete in six of these patients, with a stool frequency less than 3 stools a day. Five patients (with initial relief during treatment) reported a relapse of symptoms after cessation of this supplementation. No patients reported a worsened pattern of diarrhea and associated symptoms under *Saccharomyces boulardii* treatment.

*Effects of *Saccharomyces boulardii* on the microbiota*

The treatment with *Saccharomyces boulardii* had no effects on the fecal biostructure in healthy controls and massive impact on the stool of patients with diarrhea. The observed changes in the microbiota in these patients can best be described in terms of progressive restocking of the bioreactor.

- Increase in the absolute numbers of the main fermenting habitual bacterial groups

The concentrations of the habitual bacterial groups increased significantly with *S. boulardii* supplementation. This increase was especially obvious in case of *Roseburia* (Erec) and *Bacteroides* (Bac) groups (Table 2). The statistical significance of the differences between treated and untreated patients with diarrhea was $P<0.01$ for *Roseburia* and $P=0.014$ for the *Bacteroides* groups. The concentrations of *Roseburia* remained high, while concentrations of *Bacteroides* and *Faecalibacterium prausnitzii* started to decline after the cessation of the *Saccharomyces boulardii* therapy (Table 2).

- The decline in the substitution of habitual with occasional bacterial groups

The concentrations of all fecomucous occasional bacterial groups, which were increased in patients with diarrhea compared to healthy controls, diminished over time with *Saccharomyces boulardii* therapy (Table 2). Although the reduction of bacterial concentrations for single bacterial groups was not significant, the fact that all occasional bacterial groups reacted in the same manner, was convincing. The most profound and statistically highly significant reduction of mucotrop occasional bacterial groups was observed for

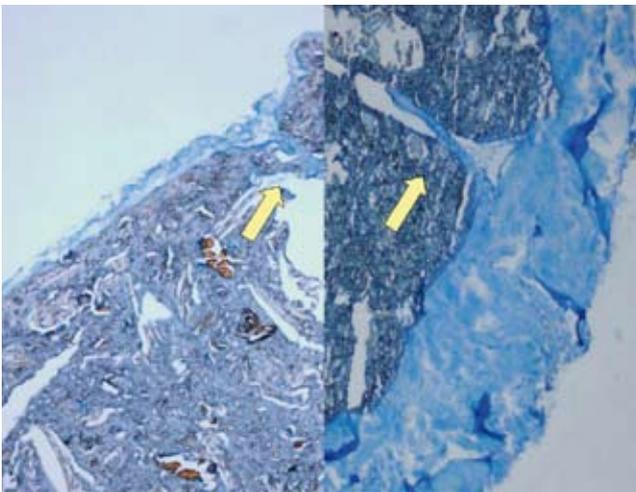


Figure 11 Mucus layer in healthy controls (left) and diarrhea patients (right), magnification x100.

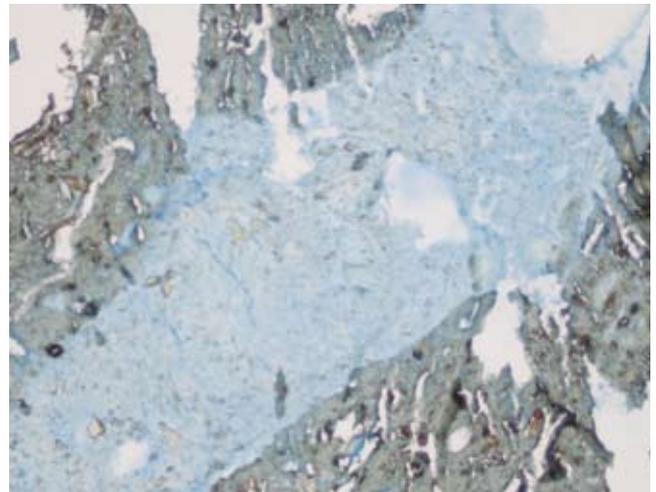


Figure 12 Mucus inclusion in the stool in form of septa in a patient with diarrhea, alcian stain x100.

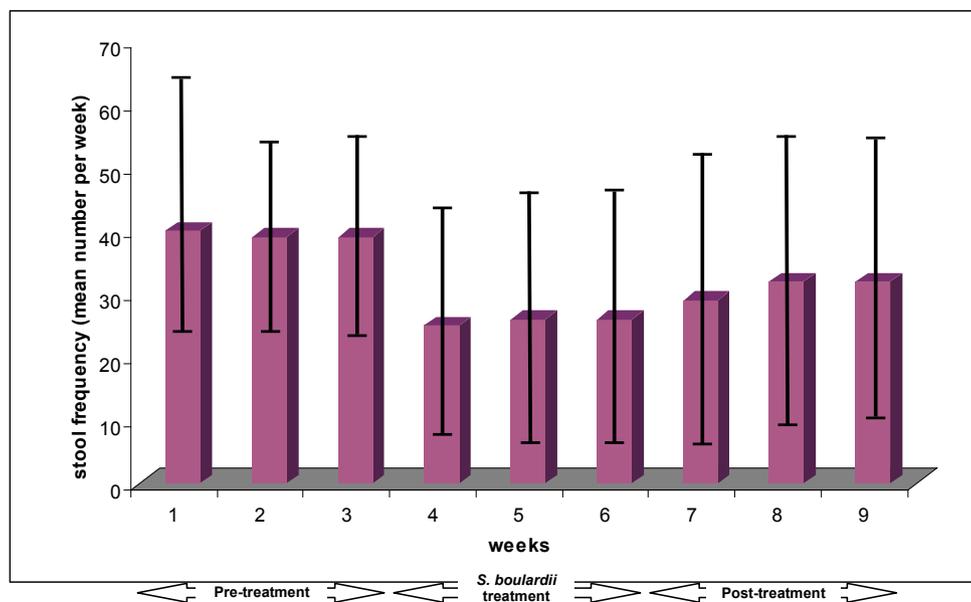


Figure 13 Influence of *Saccharomyces boulardii* on stool frequency.

Akkermansia muciniphila ($P=0.002$). The concentrations of the mucotrop occasional bacterial groups were halved under therapy with *Saccharomyces boulardii*. In 80% of patients with diarrhea, in whom *Akkermansia muciniphila* was present, the changes in concentrations of *Akkermansia muciniphila* were gradual and could be seen in individual patients over the duration of therapy. They correlated well with both clinical improvement and reduction of mucus and the watery components in stool (Tables 2 and 3). The concentration of *Enterobacteriaceae*, another mucotrop bacterial group, was not perceptibly changed following *Saccharomyces boulardii* supplementation (Tables 2 and 3). This indicates either, that the increase of mucotrop *Enterobacteriaceae* in diarrhea must have other reasons than the amounts of mucus or that *S. boulardii* directly suppresses *Akkermansia muciniphila* but has no influence on *Enterobacteriaceae*. *Akkermansia*

muciniphila concentrations increased after the cessation of the *Saccharomyces boulardii* therapy.

- Termination of the bacterial suppression and restitution of the homogeneous structure of habitual microbiota

The homogeneous uninterrupted distribution of habitual bacterial groups in the stool cylinder, characteristic for healthy controls, was restored and the striae completely disappeared in 45% of patients with diarrhea treated with *Saccharomyces boulardii*. The proportion of the stool cylinder surface occupied by mucus striae diminished significantly (Table 3).

- Reduction of the mucus production

The *Saccharomyces boulardii* supplementation had no influence on mucus in healthy controls. The median thickness of the mucus layer in patients with diarrhea decreased from $265 \pm 266 \mu\text{m}$ prior to *Saccharomyces*

boulardii supplementation to $96 \pm 118 \mu\text{m}$ during supplementation ($P=0.002$), and increased thereafter (Table 3). The occurrence of the superficial mucus layer in patients with diarrhea increased during the *Saccharomyces boulardii* supplementation and remained unchanged in the period thereafter, indicating the higher consistency of stool, which helped in the preservation of the stool cylinder (Table 3).

Discussion

The clinical relevance of data on stool microbiota is traditionally seen with a great portion of skepticism. Large amounts of information, generated by cultural and PCR based methods, do not fit in diagnostically relevant interpretations. This is partially methodologically determined. Colonic microbiota are usually perceived as a homogeneous bacterial mass filling a colonic basin. From this point of view the only reasonable approach seems to be a maximally comprehensive analysis of microbial composition. The spatially undifferentiated analysis of microorganisms inhabiting the colon revealed thousands of bacterial species and an unimaginable number of microbial combinations, which could all potentially contribute to colonic function, but thus far delivered no reliable parameter, which could be used for diagnosis or definition of normal colonic microbiota (eubiosis) or their disturbances (dysbiosis).

The oversimplified perception of colonic microbiota as a homogeneous mass is deceptive. The inhomogeneity of stool is a basic feature, which is already visible to the naked eye. Ironically, all previous investigators tried to reduce the “error of small samples” in taking larger amount of feces and homogenising it previous to analysis. While doing so, the structure- and function-specific distribution of bacteria was lost.

The colon is not just a tube forwarding, dehydrating and forming feces, it is a sophisticated high output bioreactor in which different regions fulfill highly specialized tasks: central fermenting compartment, germinal stock compartment and separating mucus layer. Our data clearly demonstrate that the presence of bacteria in different areas of the colonic bioreactor is not coincidental but directly related to the colonic function. The main stool microbiota mirror the functional compartmentalization of the colon and allow to use stool for clinical diagnosis.

Instead of detecting bacteria and bacterial genes in homogenates of feces, we tried an alternative approach allocating bacteria spatially on sections of punched-out stool cylinders with multicolor FISH. The results and the traits found with this method were astonishingly clear and reproducible, even in case of such difficult material as stool from patients with diarrhea. The colonic microbiota and disease dependent changes proved to be much easier to characterize as previously assumed.

Typical for health are:

- 1: A 5 - 60 μm mucus separating layer located mainly on the surface of the stool cylinder, no considerable inclusion of the mucus layer in the stool;
- 2: High concentrations ($>8 \times 10^{10}$ bacteria/mL) of the three main habitual bacterial groups represented by *Bacteroides*, *Roseburia* and *Faecalibacterium prausnitzii* each of which contributes 15 to 40% to the fecal mass;

- 3: Homogeneous web-like distribution and high fluorescence intensity of habitual bacterial groups both in the germinal stock and central fermenting area of the stool cylinder;
- 4: Uncertain occurrence and relative low concentrations of occasional bacterial groups represented by *Bifidobacteriaceae*, *Atopobium* etc.;
- 5: Lack of leukocytes in the mucus layer or in feces.

The features of diseased colon are typical for malfunction of any bioreactor: discharge, decontamination, and resetting. In case of chronic diarrhea, the criteria characterizing colonic dysfunction are directly related to the severity of disease and specifically include:

- growing thickness of the protective separating mucus layer. Incorporation of the mucus within stool in form of 50-500 μm thick multiple septa;
- marked decrease in absolute concentrations of habitual bacterial groups;
- disruption of the homogeneous web-like texture of habitual bacterial groups by watery ingredients, leading to stratification and microscopic appearance of “a water surface ruffled by wind”;
- suppression of the bacteria leading to declining fluorescence in the central fermenting area, with partial (less than 10%) or nearly absolute (less than 0.1%) hybridization silence of all bacterial groups,
- and substitutive increase in concentrations of occasional bacterial groups.

Four of the 5 above mentioned criteria of malfunction can be documented already by alcian stain and hybridization with three habitual bacterial groups. Single occasional bacterial groups can not be detected by FISH in each person; the surveillance of their concentrations is not applicable in each case. However, the set of 8 constitutive occasional bacteria allows, as a whole, a precise characterization of the individual situation even in case, when single occasional bacterial species are lacking.

The criteria for malfunction are gradual, directly related to the severity of the disease. They can be exactly quantified and followed longitudinally, independent from highly subjective complaints and patient’s behavior and can be evaluated in a blinded study. The ease of collection, the convenience of storage and delivery of stool samples together with the high motivation of chronically ill patients lead to high patient compliance. The immediate fixation of the material in Carnoy solution and succeeding embedment in paraffin preserves the microbiota in their native state and enables investigations of the samples in reference laboratories of choice even years later, answering emerging questions and taking advantage of newly developed FISH probes.

The structure-functional compartment analysis could be especially valuable in case of quantitative evaluation of therapeutic effects. Three weeks of *Saccharomyces boulardii* supplementation had no effects on the colonic microbiota or their structure-functional distribution in healthy persons. In contrast, the effects in patients with chronic diarrhea were profound and can be best described as a successful resetting of the colonic bioreactor. The three week substitution of *Saccharomyces boulardii* led to statistically significant reversible changes of all FISH parameter typical for diarrhea. This recovery went parallel with the clinical improvement in

14 of 20 patients indicating the efficiency of *Saccharomyces boulardii*. Future studies should help to determine whether the selection of responders from nonresponders and dosis/therapy duration finding studies can optimize the results.

Acknowledgments

The study was funded by: University Funding (Charite Hospital, Berlin University); Bundesministerium für Bildung und Forschung network "Resistance and Susceptibility to Intestinal Infections - an Integrated Network to Study the Interaction between Microbial and Host Factors".

Conflicts of interests

There are no competing interests for any of the authors.

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