Impact of humic acids on the colonic microbiome in healthy volunteers

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AIM
To test the effects of humic acids on innate microbial communities of the colon.

METHODS
We followed the effects of oral supplementation with humic acids (Activomin®) on concentrations and composition of colonic microbiome in 14 healthy volunteers for 45 d. 3 × 800 mg Activomin® were taken orally for 10 d followed by 3 × 400 mg for 35 d. Colonic microbiota were investigated using multicolor fluorescence in situ hybridization (FISH) of Carnoy fixed and paraffin embedded stool cylinders. Two stool samples were collected a week prior to therapy and one stool sample on days 10, 31 and 45.
one FISH probes representing different bacterial groups were used.

RESULTS
The sum concentration of colonic microbiota increased from 20% at day 10 to 30% by day 31 and remained stable until day 45 (32%) of hemic acid supplementation ($P < 0.001$). The increase in the concentrations in each person was due to growth of preexisting groups. The individual microbial profile of the patients remained unchanged. Similarly, the bacterial diversity remained stable. Concentrations of 24 of the 35 substantial groups increased from 20% to 96%. Two bacterial groups detected with Bac303 (Bacteroides) and Myc657 (mycolic acid-containing Actinomycetes) FISH probes decreased ($P > 0.05$). The others remained unaffected. Bacterial groups with initially marginal concentrations ($< 0.1 \times 10^7/mL$) demonstrated no response to hemic acids. The concentrations of pioneer groups of Bifidobacteriaceae, Enterobacteriaceae and Clostridium difficile increased but the observed differences were statistically not significant.

CONCLUSION
Humic acids have a profound effect on healthy colonic microbiome and may be potentially interesting substances for the development of drugs that control the innate colonic microbiome.

Key words: Fluorescence in situ hybridization; Colonic microbiota; Colonic bioreactor; Humic acids; Healthy volunteers; Oral supplementation

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Core tip: Modern patients are increasingly interested in natural medicinal products, which often not scientifically evaluated. Humins arise from organic microbial degradation and are an important mediator of microbial interactions in nature. Although used for medical indications since ancient times, no data exist on the impact of humins on the human microbiome. Our investigations in healthy volunteers show that orally applied humic acids increase the sum concentrations of preexisting colonic microbiota from 20% to 30% without changes in the bacterial diversity of the individual microbiome and may be a serious amendment/alternative to fecal transplantation or probiotics.

INTRODUCTION
All great ancient cultures were based on agriculture for which soil quality and prevention of its exhaustion were absolutely critical. Humus as an organic fecundity substrate of the earth excited thinkers from the ancient times and stimulated both solid research and charlatantry. First descriptions of medical applications can be found in Sanskrit and also ancient writings of Rome and China. Despite nearly mystic reverence and enormous interest, it is not before the early 1800s that chemical characterization and description of hemic acids took place.

Humic substances are complex organic substances of soil, which are formed in the process of humification. Humification involves natural chemical and microbial activity that transforms the dead remains of living things into humic substances. It is the second greatest organic process on earth after photosynthesis and is responsible for fossil coal, oil deposits and others. Microorganisms utilize and break down organic substances and lead to accumulation of recalcitrant molecules. When microorganisms die, they are themselves broken down and added to the recalcitrant hemic mass. The concurrent chemical-physical polymerization modifies hemic substances in an unpredictable matter. In all, the genesis of hemic substances can take hundreds or even thousands of years and leads to high variety, unique composition and extreme difficulties in characterization of these $^{1-3}$.

The growing interest of the modern society for environmental and biological welfare refreshed the attractiveness for implementation of humins. Gastroenterologists are often confronted with a wish of patients to be treated with “natural” products and asked for opinion on hemic acids. The study of the scientific literature reveals a large number of medical trials with dietary supplements of hemic acids conducted all over the world. The reported effects include different, partially incoherent properties such as anti-inflammatory and immune-stimulatory as well as analgesic, antimicrobial, antiviral/anti-HIV activity, antioxidant and even stroke protective effects $^{1-3}$. The striking eclecticism of the findings and the lack of systematic studies make it difficult to build an unbiased opinion. Furthermore hemic substances are distributed under a wide variety of trade names and descriptions in an unregulated market.

The colon is a central bio-fermenting organ degrading digestive leftovers. Since microbial activity is central in genesis and processing of hemic acids, the innate human microbial communities should be the main object on which the effects of hemic acids will be apparent. Astonishingly, we found no data to this topic in the literature. In order to close this gap, we investigated the impact of orally applied Activomin$^®$ (Pharmawerk Weinboehla, Weinboehla, Germany) on concentrations
and diversity of the human colonic microbiome. Activomin® is the only registered and standardized humic acids preparation in Germany.

MATERIALS AND METHODS

Patients, subjects and samples
Fourteen healthy volunteers from the Laboratories of Centre for Infectious Diseases, Faculty of Veterinary Medicine, University of Leipzig and Laboratory for Polymicrobial Infections and Biofilms, Charité Universitätsmedizin Berlin (24-64 years of age, mean 39 years, 5 males and 9 females) have taken 3 × 2 capsules (3 × 800 mg) Activomin® orally for 10 d followed by 3 × 1 capsule (3 × 400 mg) for 35 d. Two stool samples were collected a week prior to therapy and one stool sample on days 10, 31 and 45.

The study was approved by the ethics commission of University of Leipzig. The collection of fecal samples for fluorescence in situ hybridization (FISH) diagnosis of dysbiosis was approved by the ethics commission of the Charité University Hospital.

FISH
Colonic microbiota were investigated using FISH analysis of Carnoy fixed and paraffin embedded stool cylinders[6]. Multicolor FISH simultaneously using 3 differently stained FISH probes (C3 - orange, FITC-dobe - green, C5 - dark red) and counterstained with DAPI for DNA structures was performed on 4 μm longitudinally cut sections of punched-out stool cylinders. Sections were placed on SuperFrost plus slides.

A Nikon e600 fluorescence microscope was used. 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 number of all bacteria, C5 marked probes with a different to C3 probes specificity were used to exclude unspecific binding. 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.

Bacterial concentrations of homogeneous populations were enumerated visually in one of the 10 × 10 fields of the ocular raster corresponding to 10 μm × 10 μm of the section surface at magnification of 1000. This number was assigned to concentration of 1 × 10^9 bacteria/mL, which was most equivalent to the calculation formula, which we had used previously[6].

In case of uneven distribution of bacteria over the microscopic field, the positive signals were enumerated in ten fields of the ocular raster along the gradient of distribution and divided by ten.

Investigated bacterial groups/FISH probes
Forty-one bacterial FISH probes were applied, Table 1. The exact specification of the FISH probes and hybridization conditions are available in public resources[5]. The names of the FISH probes are listed according to abbreviations of the probeBase online resource (http://www.microbial-ecology.net/probebase/credits.asp). The Fprau probe is described in 6[6].

The FISH probes were arranged in Table 1 to four functional groups described previously: essential bacteria, individual pioneer bacteria, individual substantial and individual marginal or accidental bacteria[7].

Bacteria detected with EREC (mainly Roseburia), Bac303 (Bacteroides), Fprau (Faecalibacterium prausnitzii) probes are always present in healthy human subjects and together contribute about half of the colonic microbiome. They are obviously essential for colonic bio-fermentation.

All other bacterial groups are individual, present only in some of the subjects in substantial concentrations (mean ≥ 0.1 × 10^9/mL) or marginal concentrations (mean < 0.1 × 10^9/mL).

Four FISH probes including Bif153 (Bifidobacteriaceae), Cdif198 (Clostridium difficile), Ebac1790 (Enterobacteriaceae) and Clt135 (Clostridium lituseburensense) represent individual bacterial groups with pioneer function, which are found prevalent in newborns, after antibiotic treatment and convalescence patients, but are seldom found in low concentration in healthy persons.

Statistical analysis
Differences between groups were evaluated using the twosided t-Student U test. Data are presented as mean ± SD, P <0.05 was considered statistically significant.

RESULTS
All participants completed the stool collection, even the one man, who developed loose stools and bloating. No other side effects were reported.

Humic acids induced changes of the microbiome
Table 1 summarizes changes in the mean concentrations of single bacterial groups prior to and during supplementation with Activomin®. Bacteria in the Table 1 are arranged to sets of essential, individual pioneer, individual substantial and individual marginal bio-fermenting groups.

The mean microbial concentration after 45 d of supplementation of humic acids increased 14% in the essential groups (P < 0.01), 28% (NS) in the individual pioneer groups and 41% (P < 0.002) in the individual substantial groups. The accidental bacterial groups with initially marginal concentrations demonstrated no response to humic acids.
The response to humic acids of single bacterial groups was principally the same as in all functional sets of substantial bacteria. The concentrations of most bacterial groups within essential (2 of 3) pioneer (3 of 4) and individual substantial groups (19 of 28) increased in rates of 20% to 60%. In most cases, the increase was observed already at day 10 and continued to day 45. In groups with comparatively low initial mean concentrations (Ebac1790, Cdif198, Chis150, Eram997, Lab158, Veil223) an increase could be higher than 70% and up to 96%, but the contribution of these groups to the overall bacterial numbers was relatively low. Only the concentrations of bacteria detected with Bac303 (Bacteroides) and Mpyc657 (myclic acid-containing Actinomycetes) FISH probes decreased under humic acids supplementation, but was statistically not significant, because of the high variance and low number of probands.

The increase in concentrations of microbiota was caused by preexisting groups, and not due to emerging new microorganisms. The individual microbial profile remained constant. In none of the test persons did
the ratio of positive/negative individual groups change more than 5%.

Humic acid supplementation did not affect microbial diversity. Mean percent of substantial individual bacterial groups positive for bacteria for each person was nearly the same over time with 72%; 74%; 76%; 72% at the control days accordingly. The patterns in distribution of single bacterial groups over the stool cylinder differed depending on the species but remained the same in the mucus close transient zone and in the center of the fecal cylinder regardless of humic acids supplementation.

**DISCUSSION**

The dietary supplementation of humic acids for medical purposes and for promotion of health is deeply rooted in cultural traditions. The humus and its components are regarded as something purely biological, nature promoting and positive. However, the mechanisms how humic acids may work are purely understood. The sheer indefinite number of chemically active functional groups within the extreme complex chemical structure of humic substances makes biochemical investigations elaborate, costly and difficult to reproduce\(^1\). Even apparent effects of humic acids on the quality of soil and its microbiome remain vague and general as to ancient times and are up to now not disclosed in specific verifiable details\(^8\).

Our data first demonstrate that the humic acids are indeed global fertilizers of microbial growth as proposed by traditional view and lead to an increase of more than 30% in the mean concentrations of the colonic microbiome \((P < 0.001)\). The promotion of microbial growth involved 24 of 35 investigated substantial bacterial groups. The only investigated microbial groups that were negatively affected by humic acids were *Bacteroides* (Bac303) and *Mycobacterium* subdivision mycolic acid-containing *Actinomycetes* (Myc557). All other investigated groups were either increased or not affected.

In newborns, during stress, convalescence or disease, pioneer bacteria increase exponentially up to ranges otherwise typical for essential bacterial groups\(^9\). We did not observe such a reaction in our study. The most profound increase in concentrations to 41% \((P < 0.002)\) was that of the individual substantial bacteria. The increase in concentrations of the pioneer groups was lower \((28\%)\) and statistically not significant, indicating that host stress and convalescence of the colonic microbiome are not present. Lack of functional stress is also supported by the fact that the individual microbial profiles in all subjects remained stable over the observation period and that the patterns in distribution of bacteria over the fecal cylinder did not change under humic acids application.

The comparatively low increase \((14\%, P = 0.02)\) of the essential bacterial groups observed in our study was due to suppression of *Bacteroides*, and probably further resulted from the fact, that essential bacterial groups are already normally maximal promoted by the host and their growth cannot be endlessly boosted.

Aside of the numeric impact on the microbiome, we do not know which clinical effects humic acids promoted, since all test persons were healthy and all, except one, tolerated Activomin\(^9\) without negative or apparent positive health effects.

However, reduced diversity and concentrations of colonic microbiota were demonstrated in IBD\(^9\), IBS and non-gastroenterological diseases such as obesity, diabetes, rheumatism and multiple sclerosis\(^10\-13\). These changes in the microbiome are claimed responsible for pathogenies of multiple other diseases. To repair the disordered microbiome, fecal transplantation and probiotics have been recommended and clinically tested. However, such transfections are difficult to control and do not guarantee that the transferred microorganisms prevail, settle and proliferate in the colon\(^14\).

Humic acids exert profound effects on the colonic microbiota and may be an interesting group of substances for the development of specific drugs, which deliberately influence colonic fermentation in an inflamed colon, obesity, rheumatic and neurologic disorders.

**COMMENTS**

**Background**

Patients demonstrate increasing interest in medical treatments that are not part of mainstream medicine. Critical argumentation is important, but difficult to do when not evaluated with scientific methods. Humins are a product of microbial metabolism and an important mediator of microbial interactions and activity. As natural fertilizers humins are used for medical indications since ancient times. It is believed that the human microbiome is the main target of humic activity. However no data exist on the impact of humins on the human microbiome.

**Research frontiers**

An evergrowing number of studies demonstrate the involvement of the colonic microbiome in obesity, digestive, endocrine, inflammatory and auto-immune and neurologic disorders. Different approaches are proposed to consolidate and improve the colonic microbiome. The research hotspot is to move beyond description and to introduce substances and therapies with proven controlled graduate effects on the microbiome.

**Innovations and breakthroughs**

The presented results show, that orally applied humic acids have a profound effect on the healthy colonic microbiome. Although the effects on single microbial groups were multidirectional, the sum concentrations of all colonic microbiota increased 20% to 30%. The increase occurred in the preexisting microbial groups without changes in the bacterial diversity of the microbiome.

**Applications**

The main message of our study is, that humic acids may be an interesting substrate for the development of defined drugs, which deliberately control colonic fermentation in conditions where it is suppressed (post-antibiotics, convalescence) or altered (metabolic disorders, inflammation, obesity etc.), and are a serious amendment/alternative to fecal transplantation or probiotics.
Terminology
FISH - fluorescence in situ hybridization Cy3, FITC, Cy5, DAPI - different fluorescent dyes corresponding to orange, green, dark red and blue colours. Fluorescence in situ hybridization (FISH) combines the specific identification of microorganisms and the morphological aspect and is as a consequence especially helpful for these purposes. Each single bacterium possesses $10^7$-10^9 ribosomes of which each ribosome owns the same copy of ribosomal RNA. Some of the regions of the rRNA are strain-specific, others are universal for species, families or even kingdoms. Oligonucleotides synthesized complimentary to rRNA sequences and labelled with fluorescent dye are called FISH probes. When added to samples containing bacteria, FISH probes hybridize with the rRNA of the bacterial ribosomes. No additional enhancement of fluorescence is necessary and bacteria can be visualized directly due to the large number of ribosomes in each bacterium.

Peer-review
Although the scientific literature reveals a large number of medical trials with dietary supplements of humic acids conducted all over the world. None of the previous studies investigated effects of humic acids on the colonic microbiome.

REFERENCES