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Bacillus Clausii (O/C, SIN, N/R, T) Improves Acute Mild Colitis in Mice while *In-Vivo* Modulating Gut Microbiota

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Abstract

Background: *Bacillus clausii* is a gram-positive spore forming bacterium, used as a probiotic. Despite its wide use in acute diarrhea, little is known about its ability to modify the intestinal microbiota.

Aims: Aim of this study is to investigate the effect of *B.clausii* on a mild DSS induced acute colitis in mice evaluating gut microbiota modulation.

Methods: Mice were exposed to DSS and received *B.clausii* by gavage. Disease Activity Index (DAI) was calculated, stool samples were cultured for microbiological analysis at 3 time points, colonic inflammatory score was analyzed by histology. The direct impact of *B.clausii* on microbiota was evaluated *in vitro* using aerobic and anaerobic selective culture media.

Results: *B.clausii* slightly ameliorated mild DSS colitis in mice, with reduction of the colonic inflammatory score. *B.clausii* treatment was associated with gut microbiota modifications in stool samples. Briefly lower counts of aerobic bacteria and higher anaerobes in fecal samples were recognized compared to healthy mice. A total reduction of *Enterobacteriacee* was also observed. *In vitro* experi-

Keywords: DSS-acute colitis; Bacillus clausii; Gut microbiota.

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ments showed that *B.clausii* reduced the total count of *P.Aeruginosa* in aerobic conditions, when compared to control.

Conclusions: *B.clausii* slightly improves mild acute colitis in mice, actively modulating gut microbiota.

Introduction

Bacillus clausii is a gram-positive spore forming bacterium, used as a probiotic [1]. Amongst probiotic microorganisms, the mix of four antibiotic resistant *B.clausii* strains *(O/C, SIN, N/R, T)* represents one of the most used probiotic in clinical practice. The application of preparations containing probiotics such as *B.clausii* spores in the treatment or prevention of gut barrier impairment has been largely supported during last years. Favorable effects of *B.clausii* have been linked to several properties: resistance to commonly used antibiotics, sporogenic activity in the intestine, adherence to intestinal wall, antimicrobial and immunomodulatory activity [2-4], regulation of cell growth and differentiation, cell-cell signaling, cell adhesion, transcription and signal transduction [4], vitamins production [5] and gut protection from genotoxic agents [6].

Furthermore, *B.clausii* spores are extremely stable and capable to resist, at different terms, physical and chemical stress, such as low pH values (those present in the gastric barrier), biliary acids and at different temperatures [7-9].

These properties are linked with its ability to reach the gut alive and to exercise its pharmacological effects. The ability to adhere to intestinal walls, reducing the permeability and potentiate the local immune response, especially through secretory Immunoglobulin (IgA) has been shown for probiotics and *B.clausii* [10,11].

In human pharmacokinetic studies, *B.clausii* spores have shown to germinate in the human GI tract although the underlying mechanisms are still not completely understood [1-13].

Little is known about the effect of *B.clausii* application on species or even genus level of the gut microbiota in dysbiosis, though in clinical practice *B.clausii* is effective in preventing from dysbiosis associated symptoms such as diarrhea and abdominal pain due to antibiotic treatment [14]. Besides preventing from GI symptoms, other investigations reported immunomodulatory properties of *B.clausii*, *in vivo* [3,15].

The use of *B.clausii* has been associated to the induction of a Th1 response in nasal mucosa of patients with rhinitis, with good anti-inflammatory and immunomodulatory effect on mucosal cytokines and reduction of the pathogenic Th2 hyperactivation [15]. Starting from these observations, it could be argued that *B.clausii*, due to a potential strong Th1 response in the host, could have positive effects on experimental active murine colitis, a disease only partially resembling the human ulcerative colitis. Murine colitis, induced by DSS treatment, is in fact a good model of acute/chronic colitis, with clear signatures of Th2 colitis associated to an impairment of innate immunity mechanisms and gut microbiota alterations. Up to now, little is known about potential role of *Bacillus clausii* on clinical assessment of experimental colitis.

In the present pre-clinical study we focused on the effect of *B.clausii* on mild acute colitis in mice induced by Dextran Sodium Sulfate (DSS) with the main attention to investigate its potential microbiota modulatory property.

Matherial and methods

Induction of experimental murine chronic colitis by DSS

All animal used for this study were studied and treated in accordance with our institution's guidelines and current animal research laws, furtherly specified in the approved protocol of the present study. DSS colitis was induced on female mice C57BL/6 exposed to 1cycle of 5 days of 2.5% dextran sodium sulfate (DSS, molecular weight 36-44 kDa, MP Biomedicals, Aurora, OH, USA) in drinking water as indicated by several publications [16-18]. Animals were checked every day by measuring body weight, stool consistency and presence of fecal occult blood, as indicated [17,18]. Disease Activity Index (DAI) was calculated as described previously [18]. Animals were divided into 3 groups: 1 receiving DSS only, and 2 receiving 2 different doses of *B.clausii* by gavage daily for 5 days during the DSS cycle (dose 1 consisting in 80 mln of *B.clausii* spores and dose 2 consisting in 20 mln of *B.clausii* spores). Three parallel groups of mice not receiving DSS were used as controls. In particular the first group received only water (placebo), the second group only dose 1 consisting in 80 mln of *B.clausii* spores and the third group only dose 2 consisting in 20 mln of *B.clausii* spores). Of each group, 50% of animals were sacrificed at day 5 while all the others were sacrified at day 14th. Of each animal, plasma, faecal samples, colon, last loop of the small intestine and abdominal lymph nodes were taken for histology and molecular biology analysis. Same experimental settings are utilized on healthy mice receiving drinking water instead of DSS.

Bacillus clausii doses ad source

The probiotic preparations (Enterogermina, Sanofi-Aventis SpA, Milan, Italy) consisted of a mixture of spores of four antibiotic-resistant *B.clausii* strains named O/C, N/R, SIN and T. Each probiotic vial was stated to contain 2x10^9 CFU of *B.clausii* spores in 5 ml of oral suspension commercially available. Two doses of *B.clausii* were administered: a higher dose (dose 1) of 80 million spores and a lower dose equal to 20 million spores (dose 2), both provided in water for a total of 200 microliters by oral gavage, on day 1-5.

Dose 1: containing 80 million spores, corresponded to 200 microliters of solution available in commerce.

Dose 2: containing 20 million spores, corresponded to 50 microliters of solution available in commerce, diluted in 150 microliters of water, thus reaching a final volume of 200 microliters of solution.

Gut Microbiota assessment from feces of treated mice

Mice stool samples from all experimental groups were collected at time 0, after 5 days of treatment (time 1) and at 14º day, the end of the experiment (time 2). The specimens were then weighed and homogenized in Phosphate-Buffered Saline (PBS) to normalize for dry weight of faeces. The homogenized samples were plated in 10-fold-diluted onto TSA Agar (Becton Dickinson) and onto CNA Agar/Schaedler Agar (BioMerieux) for aerobes and anaerobes assessment. All plates were incubated at 37ºC for 24-48 h. The colonies were subsequently counted and the results are given as Colony-Forming Units (CFU)/g dry matter. Single colonies were selected for characterization of bacteria by MALDI-TOF mass spectrometry (Bruker).

Gut Microbiota assessment for *in vivo* evaluation of *Bacillus clausii* effect on fecal microbiota in experimental colitis. Mice stool samples from all experimental groups were collected at

time 0 (base line), after 5 days (time 1) and at the end of the experiment (14 days) (time 2). The specimens were weighed and collected into FECALSWAB™ with 2ml of Cary-Blair medium (COPAN) and then conducted at the microbiology laboratory to proceed to the analysis of the microbial flora.

Cultures

Culture was the first tool for the exploration of the gut microbiota and bacterial ecosystem [19-21].

We used Columbia CNA AGAR +5% sheep blood (BIOMERIEUX), a selective medium that includes colistin and nalidixic acid to inhibit gram negative growth, in order to enables important Gram-positive cocci to be recognised more readily and isolated easily from the mixed bacterial populations. Chocolate PVX AGAR (BIOMERIEUX), a variant of the blood agar plate, containing red blood cells that have been lysed by slowly heating to 80 °C, was used for the isolation of more aggressive bacteria. SCHAEDLER AGAR with Vitamin K1 and 5% Sheep Blood (BENKTON-DICKINSON), a non-selective but highly nutritive media was used for the isolation of obligate anaerobes. Each stool sample was seeded on agar media using a volumetric loop calibrated to hold 0.01 ml of sample. We used a semi-quantitative streak method. Each sample plate with its specific culture media was then incubated in Aerobic condition with 5% CO_2 for PVX agar medium and anaerobic condition for CNA and SCHAEDLER agar using an anaerobic hood. Incubation time ranged from 48h to 72 h at 37°C to identify slow growing bacterial species. After incubation, each agar plate was observed and all different colonies were identified by mass spectrometry. The microbial loads were expressed in CFU/g.

Gut microbiota characterization and bacterial strain Identification

Colony identification was performed by mass spectrometry (MALDI-TOF), permitting a rapid and effective identification of bacterial strain as reported [22]. Briefly, the MALDI-TOF uses a soft ionization mechanism, which is obtained by using a matrix added to the bacterial colonies on metal plates. Ionization is obtained by using a UV laser beam. The measurement of the time of flight into the tube to reach a detector generated spectra. Spectral comparison with data from the defined database available is automatically performed and allows identification. This is a rapid and low-cost identification method [23].

Detection of *B.clausii* in fecal samples overtime

Stool samples from healthy and DSS-colic mice were thermally inactivated by exposing the stool suspension to 80ºC for 15 minutes prior to plating (10 µl per plate) on Mueller-Hinton Agar (OXOID) and TSA AGAR (BENKTON-DICKINSON). The plates were incubated at 37ºC for 24-48 hours. The detection limit of this method was estimated to be 10 bacteria for 50 mg of stool sample. To confirm the presence of *B.clausii* strains we used a Gram staining of selected colonies and then a mass spectrometry approach with MALDI–TOF.

Histological assessment

Colon samples from scarified mice were fixed on 4% formalin and embedded in paraffin. After hydration, all sections are de-paraffined and Haematoxylin/Eosin (H&E) staining was performed. Rachmilewitz score was used for histology assessment, as shown [24].

Statistical analysis

Mean, standard deviations, standard errors were used for continuous variables. Comparisons were performed upon statistician consultant. Non-parametric tests (Willcoxon, etc) or parametric tests (T test, Chi square, Anova) were used properly.

Results

***Bacillus clausii* slightly improves mild DSS murine colitis**

The highest dose of *B.clausii* (dose 1) slightly ameliorates mild DSS colitis compared to placebo, as shown by the assessment of disease activity index (DAI) at days 2-5 ($p<005$) (Figure 1A).

At sacrifice, body weight loss and colon length did not differ significantly among different groups (Figure 1B and Figure 1C). Histologic analysis of colonic samples confirmed the mild extent of colitis, with no fibrosis. However, the group that obtained the best inflammatory score was the one treated with lower dose (Figure 1D).

***Bacillus clausii* modulates gut microbiota composition in mild DSS colitis in stool samples of mice with colitis**

B.clausii was associated to a moderate increase in total count of aerobic bacteria compared to basal time T0 ($p<0.05$), particularly at higher dose (dose 1) (Figure 2A). Similar effect was also found in healthy mice (Figure 2C). No major changes were observed in anaerobic bacteria, treated or not with *B.clausii* (Figure 2 B and D).

B.clausii was isolated from the fecal samples of mice at 5 days (data not shown) but also following its withdrawal (time 2), following the recovery procedure after heat inactivation (Figure 2 E). *B.clausii* strains produce identical white and grayish colonies on MHA (Muller Hinton Agar) as previously reported [12].

Finally, when moving to single germ analysis (Figure 3), the treatment with *B.clausii* was associated to a measurable effect on gut microbiota composition, resulting, in particular, in a significant ($p<0.05$) reduction of count of *Staphilococcus aureus*, *Clostridium innocuum* and total count of *Enterobacteriacae* like *Klebsiella oxytoca, Enterococcus casselliflavus* and *Enterococcus gallinarum*.

Other variations, which did not reach the statistical significance, included an increase in *Mycobacterium*, *Bacteroides vulgatus*, *Bacteroides uniformis*, *Lactobacillus gasseri*, *Lactobacillus johnsonii* and the increase in *Bacteroides fragilis*.

Direct modulatory effect of *Bacillus clausii* on fecal microbiota

The bactericidal effect of *B.cla*usii in relation to the other bacterial components was then evaluated in vitro.

A co-culture of *B.clausii* was set up under aerobic conditions in physiological solution.

When *B.clausii* was incubated in co-culture with *P.aeruginosa*, a significant decrease of the total count of *P.aeruginosa* itself was observed, as shown by a reduced hemolysis in culture plate (Figure 4).

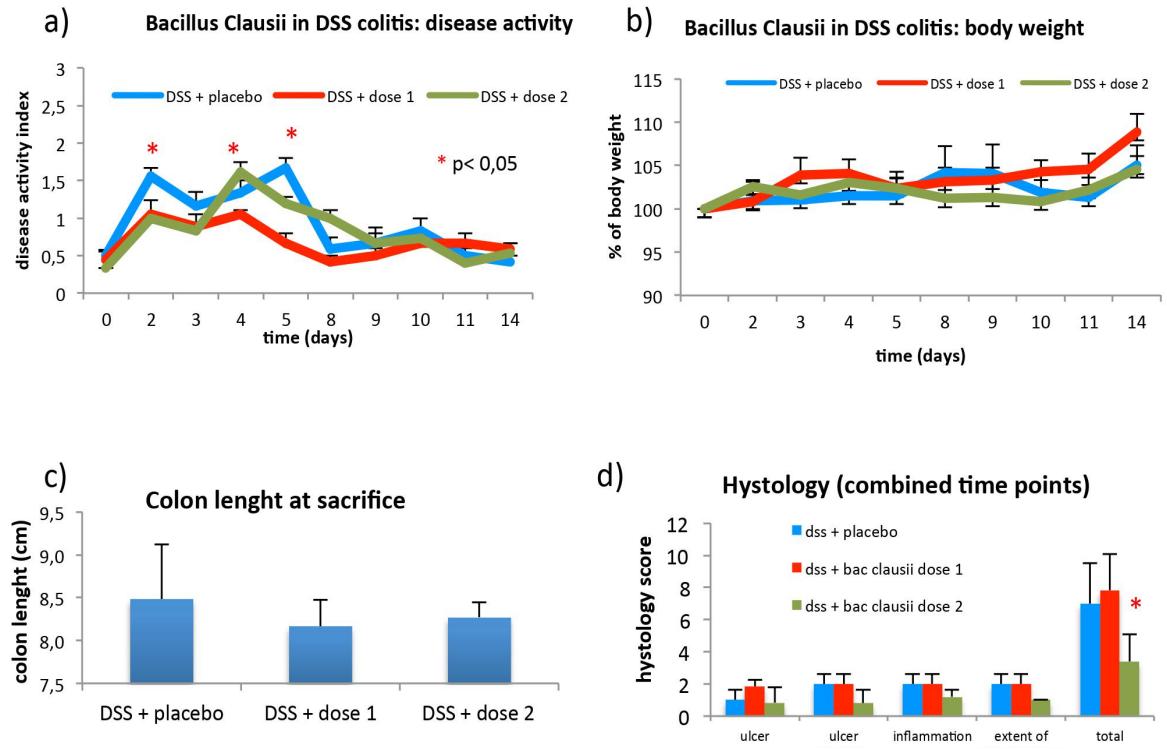
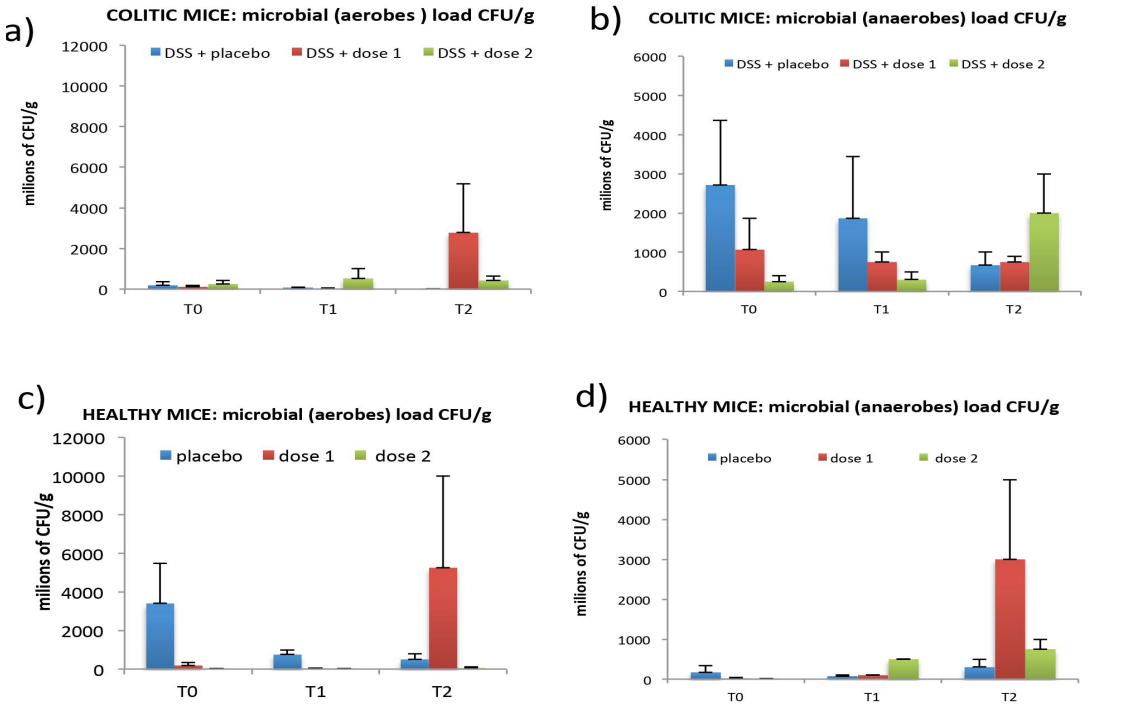


Figure 1: Bacillus clausii slightly improves mild DSS murine colitis.



e) ***Detection of B.clausii in fecal samples of treated mice:*** B.clausii strains produce identical white and grayish colonies on MHA and TSA agar respectively.

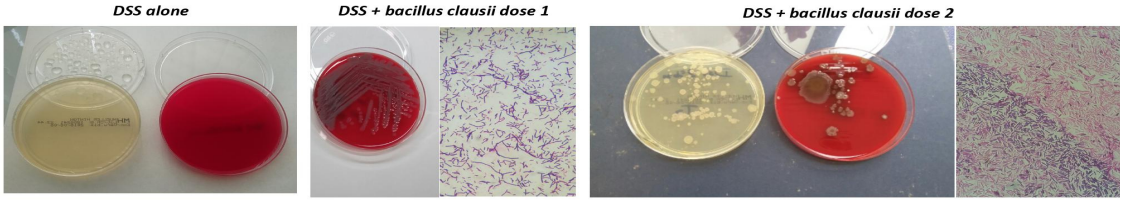


Figure 2: Bacillus clausii modulates gut microbiota in mild DSS murine colitis, increasing aerobes microbial counts in colitic mice. A and B: Colitic mice (DSS) with or w/o bacillus clausii. C and D) healthy mice. D) Detection of B. clausii in fecal samples of treated mice.

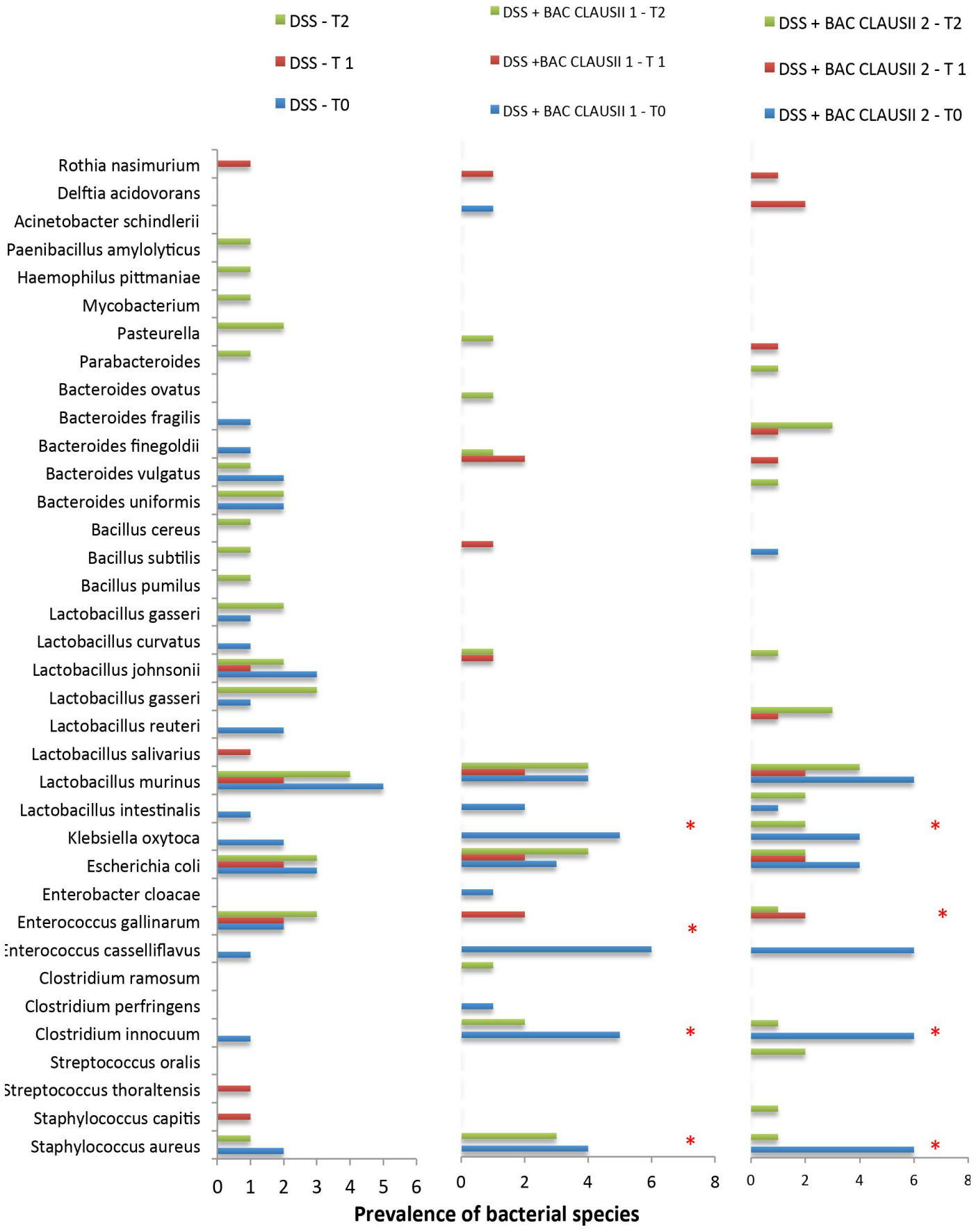


Figure 3: Species variation in course of DSS colitis and treatment with Bacillus clausii.

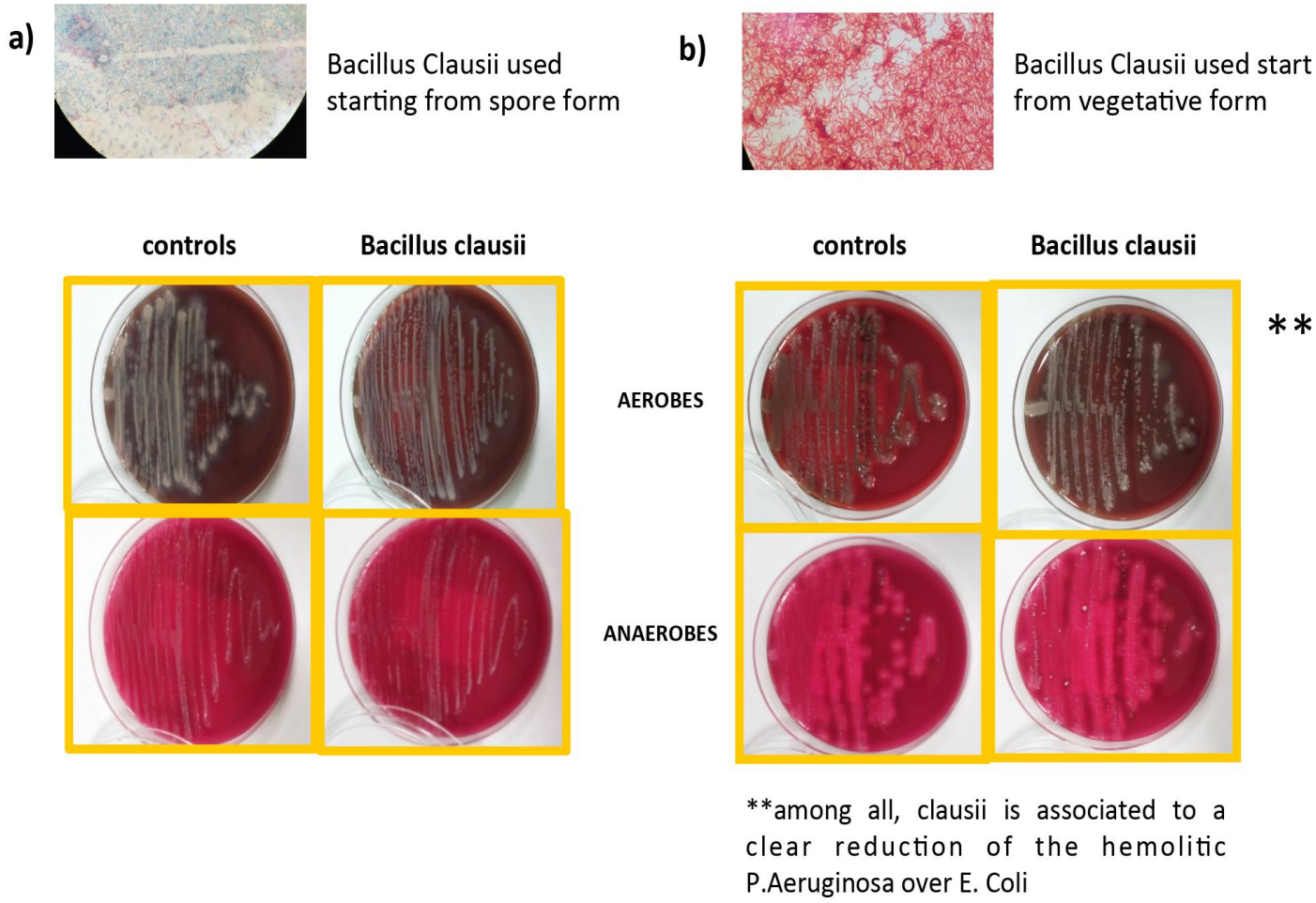


Figure 4: Direct modulatory effect of Bacillus clausii on fecal microbiota.
(a) Bacillus clausii used in a spore form.
(b) Bacillus clausii used in a germinated form.

Discussion

To our knowledge, the present paper assesses for the first time that the probiotic *B.clausii* (strains O/C, SIN, N/R, T) modulates the gut microbiota while acting as a mild modulator of subacute murine colitis. On one hand, these observations are consistent with the well described immunomodulatory properties of *B.clausii*, described in literature. On the other side, it opens new possibilities to study further usage in conditions that could benefit from its these pharmacological effects.

In the present experiments we aimed to rule out potential toxic effects of *B.clausii*. Therefore we used relatively low doses of *B.clausii,* that allowed us to detect, at the same time, a slight improvement of colitis and a significant microbiota modulatory effect in a dose dependent manner.

The combined immunological and microbiological effect of this probiotic is supported by the *in vitro* findings. In facts *B.clausii* itself, when not given orally but incubated on fecal samples of mice not previously exposed to it, only exerts a minor effect, including a decrease of the total count of *P. Aeruginosa* over *E. Coli.* On the other hand, when given orally during an acute phase of colitis, it is associated to an overall increase in the total count of aerobic bacteria, modulating the microbiota of colic mice toward the microbiota of the healthy phenotype.

Furthermore, the use of *B.clausii* is associated to a significant reduction of potentially harmful commensals like *Staphilococcus aureus*, *Clostridium innocuum* and total count of *Enterobacteriacae* (*Klebsiella oxytoca, Enterococcus casselliflavus* and *Enterococcus gallinarum)*.

Lat but not least, the presence of *B.clausii* in stool samples of colitic mice in form of spore, as demonstrated by the growth capacity following heat inactivation, suggests that *B.clausii* remains within the colon up to 9 days from the beginning of therapy. This data suggest that in the intestine *B.clausii* acts not only as vegetative bacteria but also as a spore form.

The last aspect that needs to be addressed is that *B.clausii* seems to ameliorate the overall histology of colitic mice when given at lower dose. However, this effect was observed during the recovery phase of the colitis, when the clinical symptoms were almost silent in both control and treated mice. We believe that these findings have to be discussed in the light of the mucosal healing phase following the acute phase of colitis. Further investigations are needed to fuel these discussions.

This paper has several limitations. First of all we did not perform more mechanistic analysis about the cytokine modulation by *B.clausii* at the intestinal level that is associated to gut microbiota modulation. We believe that this topic is worth to be investigated in dedicated trials. The experimental setup, to answer these questions, would need blood and biopsy samples at the time of animal sacrifice, in order to perform the analysis of the inflammatory cytokine expression on both serum and tissue samples.

Furthermore, we performed the analysis of the microbiota through "culturomics" instead of metagenomics. We believe that this is not a real limitation, as also "culturomics", have shown to deliver valid results in the investigation of the modulation of the gut microbiome in health and disease models [25]. The culturomics choice over the genomic is more sensitive for "quantitative" data on the overall effect on B.clausii; furthermore we wanted to assess also the "growth" of B.clausii itself over its vegetative form in order to induce a microbial change.

Surprisingly, although we clearly detected *B.clausii* in treated mice, the amount of the bacterium recovered in the feces was low. That suggests that *B.clausii* acts not just as a gut microbe covering a niche in the intestine, but potentially induces immunological changes that are able to reinforce its "ecological" effects.

The present paper strongly encourages further studies to investigate its effect on the gut microbiome in health and disease models, in order to define new therapeutic areas for this probiotic besides the treatment of dysbiosis associated symptom like diarrhea.

Institutional review board statement

The study was reviewed and approved by the Catholic University Institutional Review Board.

Institutional animal care and use committee statement

All experiments were approved by the Local Ethics Committee for Animal Research Studies at the UNIVERSITA' CATTOLICA DEL SACRO CUORE of Rome (Protocol Number 15699/15 - 08/07/2015 and the Superior Service Ethics Commission Animal Health and the Ministry of Health 1075/2015-PR09/10/2015). All animal used for this study received "humane care" and all experiments complied with our institution's guidelines and current animal research laws, as well specified in the approved protocol of the present study.

Conflict-of-interest statement

Antonio Gasbarrini, Franco Scaldaferri and Loris Riccardo Lopetuso have been lecturer for SANOFI. This project has been conducted with an unrestricted grant provided by SANOFI to UNIVERSITÀ CATTOLICA DEL SACRO CUORE of Rome, under the supervision of Franco Scaldaferri

Data sharing statement

Technical appendix, statistical code, and dataset available from the corresponding author at francoscaldaferri@gmail.com.

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