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Intestinal Fungal Dysbiosis Associates With Visceral Hypersensitivity in Patients With Irritable Bowel Syndrome and Rats

Short title: Fungi in irritable bowel syndrome (IBS)

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Author contributions: RvdW developed the concept; WJdJ, JS and RvdW designed experiments; SB, OW, SEH and MF conducted research; DMJ and AAM collected and provided human samples; all authors contributed to data analysis (ITS and 16S analysis and statistics GR, EL, HHdW and FHJS); SB and RvdW wrote the paper; GR, EL, WJdJ and JS critically revised the manuscript for important intellectual content; all authors read and approved the final manuscript.

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Background & aims: Visceral hypersensitivity is one feature of irritable bowel syndrome (IBS). Bacterial dysbiosis might be involved in activation of nociceptive sensory pathways, but there have been few studies of the role of the mycobiome (the fungal microbiome) in development of IBS. We analyzed intestinal mycobiomes of patients with IBS and a rat model of visceral hypersensitivity.

Methods: We used internal transcribed spacer 1-based metabarcoding to compare fecal mycobiomes of 18 healthy volunteers with those of 39 patients with IBS (with visceral hypersensitivity or normal levels of sensitivity). We also compared the mycobiomes of Long Evans rats separated from their mothers (hypersensitive) with non-handled (normally sensitive) rats. We investigated whether fungi can cause visceral hypersensitivity using rats exposed to fungicide (fluconazole and nystatin). The functional relevance of the gut mycobiome was confirmed in fecal transplantation experiments: adult maternally separated rats were subjected to water avoidance stress (to induce visceral hypersensitivity), then given fungicide and donor cecum content via oral gavage. Other rats subjected to water avoidance stress were given soluble β-glucans, which antagonize C-type lectin domain family 7 member A (CLEC7A or DECTIN1) signaling via spleen associated tyrosine kinase (SYK), a SYK inhibitor to reduce visceral hypersensitivity, or vehicle (control). The sensitivity of mast cells to fungi was tested with mesenteric windows (ex vivo) and the human mast cell line HMC-1.

Results: α diversity (Shannon index) and mycobiome signature (stability selection) of both groups of IBS patients differed from healthy volunteers, and the mycobiome signature of hypersensitive patients differed from that of normally sensitive patients. We observed mycobiome dysbiosis in rats that had been separated from their mothers compared with non-handled rats. Administration of fungicide to hypersensitive rats reduced their visceral hypersensitivity to normal levels of sensitivity. Administration of cecal mycobiomes from rats that had been separated from their mothers (but not non-handled mycobiome) restored hypersensitivity to distension. Administration of soluble β-glucans or a SYK inhibitor reduced visceral hypersensitivity, compared with controls. Particulate β-glucan (a DECTIN-1 agonist) induced mast cell degranulation in mesenteric windows and HMC-1 cells responded to fungal antigens by release of histamine.

Conclusions: In an analysis of patients with IBS and controls, we associated fungal dysbiosis with IBS. In studies of rats, we found fungi to promote visceral hypersensitivity, which could be

reduced by administration of fungicides, soluble β-glucans, or a SYK inhibitor. The intestinal fungi might therefore be manipulated for treatment of IBS-related visceral hypersensitivity.

Key Words: mycobiota, dectin-1, immune response, yeast

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Introduction

Irritable bowel syndrome (IBS) is a highly prevalent, stress related functional gastrointestinal disorder that is characterized by the presence of abdominal pain with altered bowel habits. Although IBS is a heterogeneous disorder, abdominal pain is a common denominator in all patients and a major unmet clinical need.[1-4] Increased sensitivity to distension of the gastrointestinal tract, so called visceral hypersensitivity, is observed in ~50% of patients and hypothesized to be an underlying pathophysiological mechanism.[5] In animal models, gut mucosal mast cells and their mediator histamine were shown to mediate visceral hypersensitivity. These results were recently confirmed in clinical studies were part of the IBS patients responded favorably to histamine receptor antagonists ketotifen and ebastine.[6-9] How mast cells become activated is only partly elucidated. In acute stress, peripheral corticotrophin releasing factor (CRF) was shown to trigger mast cell degranulation and consequent gut epithelial barrier dysfunction and visceral hypersensitivity.[10, 11] In contrast, continued post-stress visceral hypersensitivity, which was also mast cell dependent, could not be reversed by CRF-receptor antagonist in a rat model.[12] Moreover, clinical trials with such antagonists were unsuccessful.[13, 14] Due to gut barrier dysfunction, that was also evidenced in IBS patients, microbial antigens normally confined to the gut lumen become exposed.[11, 15] Indeed, several Toll-like receptors relevant for bacterial recognition as well as antimicrobial peptides were shown to be upregulated in IBS, and bacterial microbiome dysbiosis of the gut was broadly investigated as a peripheral trigger for complaints.[16, 17] However, evidence linking bacteria to IBS complaints has been circumstantial and sometimes even conflicting.[17, 18] Fungi are a minor component of the gut microbiota.[19] This may explain why the possible role of fungi in IBS, except for a small set of early and methodologically limited studies[20], was largely ignored so far. Despite low abundance, recent evidence indicated that resident fungi can play a role in inflammatory bowel disease.[19, 21-24] In addition, it was shown that fungi/fungal antigens are activators of mast cells.[25, 26] Together this led us to assess the possible role of fungi (i.e. the mycobiome) in abdominal pain in a cohort of IBS patients and an animal model for IBS.

We used an Internal Transcribed Spacer (ITS)-1 based barcoding approach to demonstrate gut mycobiome dysbiosis in hypersensitive IBS patients compared to normally sensitive patients and healthy volunteers. Next, we evaluated whether gut fungi can be a direct cause for abdominal pain by using the rat maternal separation model of stress-induced IBS-like visceral

hypersensitivity. Fungicide treatment was able to reverse visceral hypersensitivity and fecal transplantation studies showed functionally relevant mycobiome differences between maternal separated and non-handled rats. Altered mycobiome composition in maternal separated rats was confirmed by mycobiome analysis. Finally, we demonstrated that host recognition of fungi via the Dectin-1/Syk signaling pathway is essential for post stress visceral hypersensitivity.

Material and Methods

Patient characteristics and ethics statement

Patient characteristics are given in Table 1. The IBS and healthy volunteer population included are subgroups of the Maastricht-IBS cohort, comprising 540 IBS patients and 205 healthy controls. The patients represent a mixed population from primary to tertiary care with an established clinical diagnosis of IBS according to the Rome III criteria. Age, gender, medication use, mean anxiety, depression and symptom scores do not differ when compared to the total IBS cohort (data not shown). Depression prevalence scores ≥ 8[27] in healthy volunteers, hypersensitive IBS and normally sensitive IBS were 0%, 21.1% and 20.0% respectively, prevalence scores for anxiety were 16.7%, 31.6% and 30%. Dietary intake as assessed by a validated food frequency questionnaire was available for a subgroup of patients and all controls (supplementary Table S1). No differences were found between overall energy intake, and intake of total protein, fat, carbohydrates and fibres between hyper-and normosensitive patients. The reported intake was however significantly lower in the total group of IBS patients (hyper- plus normosensitive) when compared to healthy volunteers (data not shown). Subjects included in the cohort gave written informed consent prior to participation. The study protocol had been approved by the Maastricht University Medical Center Committee of Ethics and is executed according to the revised Declaration of Helsinki (59th general assembly of the WMA, Seoul, South Korea, October 2008). The Maastricht IBS cohort study has been registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT00775060).

Animals and ethics statement

Long-Evans rats (Harlan, Horst, The Netherlands) were bred and housed at the animal facility of the Academic Medical Center (Amsterdam, The Netherlands) under conditions of controlled light (06:00–18.00 h), temperature (20–22 ºC) and humidity (45%). Non-handled and

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maternal separated rats were always bred in the same room but never shared the same cage. Importantly, individually ventilated cages were only used during the post anti-fungal treatment and repopulation period in experimental protocol 2. During all experiments rats were housed in groups of 4-6 animals. Water and food were available ad libitum. All animal procedures were conducted in accordance with the institutional guidelines and approved by the Animal Ethical Committee of the AMC/University of Amsterdam (reference protocol number 100998).

Results

Mycobiome differences exist between healthy volunteers, hypersensitive IBS and normally sensitive IBS patients.

We compared the fecal mycobiome of healthy volunteers, hypersensitive IBS patients and normally sensitive IBS patients. Figure 1A depicts the 30 most predominant species and shows that the human mycobiome is dominated by two yeasts; *Saccharomyces cerevisiae* and *Candida albicans*. In healthy volunteers, their combined presence was 57% of total reads. In IBS these species were even more predominant and added up to 76% and 83% in hypersensitive and normally sensitive IBS patients respectively. Statistical analysis on the combined two species showed no difference when comparing the two IBS subgroups with each other (Supplementary Figure 1) but we did observe statistical differences between healthy volunteers and IBS subgroups. This may explain the loss of mycobiome diversity in patients as calculated by the Shannon index that combines richness (number) and evenness (relative abundance) of species.[28] Figure 1B shows significantly higher Shannon diversity in healthy volunteers when compared to both of the IBS subgroups (hypersensitive and normally sensitive IBS, $P<0.01$ and $P<0.001$ respectively).

Bray Curtis dissimilarity of ITS data did not reveal well defined clusters to separate healthy volunteer and IBS sub-groups. However, a similar negative result was obtained when analyzing the 16S rRNA gene data set; Bray-Curtis analysis did not reveal overt differences in bacterial microbiome composition (Supplementary Figure 2). To further explore potential differences between healthy controls and IBS patients we then addressed the high dimensionality of the mycobiome with a stability selection approach.[29] With this, data are repeatedly subsampled and variable selection, via elastic net classification model, is performed on each subsampled dataset. Figure 1C depicts the computed weights of species selected frequently when comparing

hypersensitive IBS patients to healthy volunteers. These species are truly associated with outcome and indicate that inter group mycobiome differences exist. The accuracy of the stability selection approach was confirmed by constructing a receiver operating characteristic curve (Figure 1D). Subsequent computations of the area under the curve (AUC) showed outstanding (i.e. AUC>0.9) discriminating quality. We also assessed differences between normally sensitive IBS and healthy volunteers, and hypersensitive IBS and normally sensitive IBS. The most robust markers of inter group differences are depicted in Supplementary Figure 3A-B. Corresponding receiver operating characteristic AUCs indicated good discriminating quality for these comparisons (Supplementary Figure 3C-D). Thus, there is altered community composition of the mycobiota in hypersensitive IBS when compared to healthy volunteers and normally sensitive IBS patients.

Essential role of fungi in a rat model of stress-induced IBS-like visceral hypersensitivity.

To further examine the possible role of fungi, we used the well-established rat maternal separation model.[7, 8, 12] We confirmed that water avoidance stress at adult age induces visceral hypersensitivity in maternal separated Long Evans rats whereas non-handled rats remained normally-sensitive (see vehicle treated rats in Figure 2A). To test the involvement of the mycobiome, two fungicides (fluconazole or nystatin) were administered to maternally separated rats for a three week period prior to water avoidance. Both fungicides independently prevented the occurrence of post-stress visceral hypersensitivity compared to vehicle in maternal separated rats (Figure 2A). Non-handled rats were not affected by fungicide treatment.

The functional relevance of the gut mycobiome was confirmed in post water avoidance fungal depletion and fecal transplantation experiments (setup depicted in Figure 2B). Three separate groups of adult maternally separated rats were first subjected to water avoidance stress which caused post stress visceral hypersensitivity in all groups (Figure 2C). All rats were then treated with a combination of fluconazole and nystatin, resulting in amelioration of visceral hypersensitivity. Next, donor caecum content was administered by oral gavage. Prior to collection of this transplantation material, donor rats were subjected to colonic distension protocols to ascertain correct post water avoidance visceral sensitivity status (Supplementary Figure 4). Recipient group 1 was then supplemented with pooled caecum content of fungicide treated maternally separated donors. This procedure did not restore visceral hypersensitivity. In

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contrast, in recipient group 2 the hypersensitive phenotype was re-induced when rats were gavaged with caecum content of maternally separated donors that were not treated with fungicides. In recipient group 3, donor material obtained from non-fungicide treated non-handled rats did not induce visceral hypersensitivity. Thus, fungicides reduce visceral hypersensitivity and transplantation of donor caecum content from non-fungicide treated maternally separated rats is able to re-establish the hypersensitive phenotype. These experiments provide strong evidence for a causal role of fungi in the etiology of visceral hypersensitivity and also suggest that relevant differences exist between the mycobiome of non-handled and maternally separated rats.

The fecal mycobiome of maternal separated rats differs from non-handled rats.

Similar to the human mycobiome, we addressed possible differences between groups in our rat model by ITS1 based metabarcoding. We first confirmed a normal visceral sensitivity status of non-handled rats and post water avoidance hypersensitivity in maternally separated rats that was reversed upon fungicide treatment (Figure 3A). Fecal pellets of these rats were then used for mycobiome analysis. The Shannon diversity index indicated significantly higher alpha diversity in non-fungicide treated maternally separated rats compared to non-treated non-handled rats and fungicide treated maternally separated rats (Figure 3B). Beta diversity (i.e. compositional difference between samples) was then determined by unsupervised cluster analysis using Bray-Curtis dissimilarity. This approach revealed distinct clusters corresponding to non-handled, maternally separated and fungicide treated maternally separated rats as depicted in the (left side) dendrogram of Figure 3C. The heat map in this figure shows the relationship between clustering and prevalence of the 20 most abundant fungal species. Together, these results confirm altered mycobiota composition in maternal separated rats that was already suggested by the fecal transfer experiments. In contrast, Bray-Curtis dissimilarity analysis of 16S rRNA gene data sets did not reveal well defined clusters to separate non-handled rats from maternal separated and fluconazole/nystatin treated maternal separation rats (Supplementary Figure 5). This lack of 16S-based separation between pre- and post-fluconazole/nystatin treatment samples suggests that fungicide-induced analgesia in maternal separated rats was not driven by consequential bacterial microbiome changes.

Mycobiome dissimilarities were further emphasized by stability selection results shown in Supplementary Figure 6. Depicted are the fungal biomarkers contributing to inter group

differences when comparing non-handled to maternally separated rats. Finally, to further strengthen the notion of strong dissimilarity between groups, we applied a co-regularized spectral clustering algorithm to the dataset.[30, 31] A heatmap plot of the resulting co-occurance matrix is depicted in Figure 3D. Seven out of 8 of the non-handled rats fell within a separate cluster to the exclusion of the maternally separated rats and fungicide treated maternally separated rats. Within clusters, individual rats share similar mycobiome composition.

Comparable to earlier findings[22, 23], a number of species identified in rat feces was also observed in chow, but clear differences do exist (Figure 4A). Furthermore, while being on the same diet, non-handled and maternal separated rats displayed different relative species contributions (examples in Figure 4B-E), suggesting that chow is not the most important source of gut fungi. In Supplementary Table 2 we used the results of human and rat stability selections (depicted in Figure 1C and Supplementary Figure 6A respectively) to compare human and rat mycobiome composition on species level. Analogous to publications on the bacterial microbiome in other rodent models[32] there is only partial overlap between human and rat mycobiota. Comparison at fungal class level confirmed partial overlap (Supplementary Figure 7). Thus, our rat model can be used for proof of principle studies where experimental manipulations address the possible role of fungi in post stress visceral hypersensitivity but other fungi may be involved in IBS pathophysiology.

Fungal recognition via the Dectin-1/Syk signaling pathway is important in post water avoidance visceral hypersensitivity.

We next sought to elucidate the mechanism by which fungi could affect the IBS phenotype. The C-type lectin receptor family is important in the recognition of fungi by the immune system. Several of these receptors signal through Syk-dependent pathways.[33] The use of a specific Syk inhibitor resulted in reversal of visceral hypersensitivity after water avoidance (Figure 5A). An important Syk recruiting C-type lectin receptor is Dectin-1 that recognizes the fungal cell wall component particulate β-glucan, an interaction that can be antagonized by soluble β-glucans.[34] High dose soluble β-glucans (50 mg/kg, applied 3 times within a 24 hour timeframe) prevented increase in visceral sensitivity after water avoidance stress, whilst vehicle and low dose (20 mg/kg) soluble β-glucan administration had no effect on hypersensitivity (Figure 5B). Next we investigated whether treatment with soluble β-glucans can also reverse visceral

hypersensitivity. Starting directly after water avoidance stress, we administered the 50 mg/kg dose during a 1 week treatment protocol (b.i.d gavage). Whereas vehicle treated rats remained hypersensitive, soluble β-glucans reversed post water hypersensitivity to distension (Figure 5C). Although involvement of ligand-receptor interactions other than β-glucan/Dectin-1 cannot be excluded, these findings do indicate the relevance of fungal recognition in the genesis of visceral hypersensitivity. Since the role of mast cells and histamine in preclinical models and part of IBS patients is now firmly established[6-9], we next sought to confirm earlier reports on fungi/fungal-antigen induced mast cell activation.[25, 26] We used Texas-red labeled avidin to visualize degranulating mast cells in gut mesenteric windows. In contrast to incubation with control bovine serum albumin solution, particulate β-glucans induced mast cell degranulation (Supplementary Figure 8A-C). Moreover, when the Dectin-1 expressing mast cell line HMC-1 was incubated with heath inactivated *C. albicans* (2.5:1 ratio), histamine release-levels equaled those of 50 µg/ml compound 48/80 (Supplementary Figure 8D-E). Thus, we conclude that mast cells are indeed equipped to recognize fungi and respond with histamine release.

Discussion

So far, investigations on the possible role of gut microbial communities in IBS almost exclusively focused on bacteria. Based on recent evidence that fungi can aggravate disease severity in colitis[19, 21-24], we assessed the role of the gut mycobiome in IBS. We observed altered mycobiome composition in patients, which let us to assess the role of fungi in the maternal separation model for stress induced visceral hypersensitivity. Fungicide treatment of hypersensitive rats reversed the enhanced response to colonic distension. Subsequent fecal transplantations showed that only the maternal separation mycobiome was able to reconfer visceral hypersensitivity to fungicide treated maternal separation rats. Experimental evidence on the role of the Dectin-1/Syk pathway indicated that direct activation of the host immune system by fungal antigens is relevant for visceral hypersensitivity. Finally, we showed that mast cells are capable of histamine release upon stimulation with fungi.

Our earlier investigations in the maternal separation model established an important role for mast cells and the histamine-1-receptor.[7, 8] These results were confirmed in clinical trials[6, 9], but triggers for mast cell degranulation remained elusive. In rat, it was shown that CRF receptor

antagonism can prevent acute stress-induced mast cell degranulation and resulting visceral hypersensitivity.[10, 11] In contrast, post stress hypersensitivity to distension, although it was also mast cell dependent, could not be reversed by CRF receptor antagonist.[12] Our present data suggest that continued visceral hypersensitivity is driven by cellular recognition of fungal β-glucans that are normally confined to the gut lumen. Although we did not investigate mechanisms involved in the translocation of these antigens, an earlier publication by Ait-Belgnaoui *et al.* indicated that barrier dysfunction is a prerequisite for stress-induced hypersensitivity to distension. Using a partial restraint model it was shown that treatment with a tight junction blocker or a specific myosin light chain kinase inhibitor not only preserved barrier integrity under stress conditions, but also prevented the development of visceral hypersensitivity.[35] Although not formerly shown, the authors suggested that stress-induced opening of tight junctions favors the uptake of luminal antigens, leading to activation of mucosal immune cells and subsequent sensitization of sensory afferents. Gut barrier dysfunction was repeatedly described in IBS patient studies[11, 15] and the maternal separation model[36, 37], indicating that similar mechanism may explain our results. Taken together, the above data suggest that post stress visceral hypersensitivity may be the result of a two-stage process (Figure 6). Upon acute stress (phase 1), peripheral CRF triggers mast cell degranulation and, consequently, afferent activation and barrier dysfunction. The latter facilitates uptake of fungal antigens like particulate β-glucans, and renewed mast cell activation leading to a 'self-sustaining loop' of continued barrier dysfunction and visceral hypersensitivity (phase 2). Importantly, fungi are considered gut commensals but may become life threatening pathogens in immunocompromised individuals. Successful anti-fungal immunity can involve innate and adaptive responses.[38, 39] Overt inflammation is absent in IBS and the rat maternal separation model.[8, 40] Therefore, our data on the role of the Dectin-1/Syk pathway suggest a subtle but effective innate anti-fungal immune response that occurs at the cost of chronic pain. Regarding the proposed perpetual nature of phase 2, previous results indeed showed that maternal separated rats, when exposed to 1 hour of water avoidance stress, remained hypersensitive to distension for at least 1 month afterwords.[12]

When treating rats with fungicides it is conceivable that this will also lead to bacterial microbiome changes that can be held responsible for the observed in vivo analgesic effect. However, 16S microbiome analysis did not show differences between pre- and post-fungicide treated maternal separation rats. In addition, our experiments on the role of fungal recognition via

the Dectin-1/Syk pathway suggested that not bacteria but fungi drive post stress visceral hypersensitivity. This is also relevant for the fecal transfer experiments where only transfer of non-fungicide treated maternal separation caecum content was capable of restoring visceral hypersensitivity in fungicide treated maternal separated rats. These experiments suggested that a specific mycobiome is required and comparison of maternal separated and non-handled fecal samples indeed showed separation-induced mycobiome dysbiosis. This result confirmed our observations in human where we found mycobiome differences between healthy volunteers and hypersensitive IBS, and between normally sensitive and hypersensitive patients. How a difference in mycobiome composition is relevant in relation to immune activation during fecal transfer was not addressed here. However, in a large set of clinical isolates, Odabasi et al. showed a wide range of (1→3)-β-d-glucan levels in fungal culture supernatants.[41] Particulate β-glucans are known to ligate Dectin-1 and possibly increased numbers of high level β-glucan expressing species in donor feces of maternal separated rats can explain our results. Moreover, even under equal expression of β-glucans the exposure to the fungal cell surface can vary among different morphological forms and species.[39] Relevant differences may also occur on strain level. In 2007, two papers with seemingly opposite results were published in Nature Immunology. One investigation showed that Dectin-1 was essential for controlling systemic infection with *C. albicans* in mice, the other found that Dectin-1 was not required.[42, 43] In a follow up paper, these contradicting findings were shown to result from the usage of different strains of *C. albicans*.[44] In this case the authors showed variations in adaptability to the immunological status of the host, resulting in substantial differences in cell wall architecture and innate immune recognition. Strain differences may also be relevant in the mycobiome specific visceral hypersensitivity shown in our repopulation experiments, but were not addressed in our mycobiome assessment.

The reason for mycobiome dysbiosis in maternal separated rats remains elusive at this stage but may relate to changes in dam-pup interactions in the postnatal period and/or altered HPA-axis responsiveness that were described in this model. Their effect on the mycobiome was never investigated but both were shown to affect bacterial microbiome composition.[45, 46] Diet was also evidenced to be an important regulator of mycobiome diversity[47] but all rats were on the same chow. In contrast, dietary differences within and in between groups may explain why mycobiome dissimilarity between healthy volunteer and IBS subgroups was less pronounced than in rat. In mouse studies, antibiotics were also shown to induce mycobiome perturbations.[48] Thus,

variable use of antibiotics may be another important factor leading to increased mycobiome heterogeneity within healthy volunteer and patient groups. To avoid short term antibiotics-induced interferences healthy volunteers and patients were only included when not having used antibiotics and anti-fungals for a period of at least 3 months before sample collection. Yet, uncertainties like earlier antibiotics use and diet may explain why differences between human sample groups could be shown by stability selection but not by the unsupervised Bray-Curtis dissimilarity analysis that was used in rat data. Similar considerations may explain the absence of bacterial microbiome differences between normal control and patient groups. Clearly a future replication study in a separate cohort will be needed to confirm the correlation between IBS and mycobiome dysbiosis.

Our data on fungal α-diversity showed contradicting results between rat and human. The Shannon diversity index indicated higher mean species diversity in healthy volunteers compared to IBS subgroups. In rat, not the normal but the IBS-like phenotype instead was associated with highest α-diversity. This discrepancy possibly arises due to the relative overabundance of *S. cerevisiae* and *C. albicans* in all human samples. The observed shift in their presence may have impacted α-diversity in IBS samples and such over representation of a limited set of fungal species was not observed in rat. In line with our observations, Sokol *et al.* found higher fungal α-diversity in fecal samples of healthy volunteers compared to inflammatory bowel disease patients.[24] DSS colitis in mouse however, showed no significant differences in α-diversity between normal and colitic mice.[23] Another factor contributing to discrepancy in human/rat α-diversity may be that, similar to the rodent bacterial microbiome, there is only partial overlap between rat and human fecal mycobiota. This also complicates predictions on the translational value of the current investigations in rat. Similar considerations apply to pre-clinical microbiome-related investigations in colitis models[32] Nevertheless, a role of the microbiome in inflammatory bowel disease is now widely accepted.

In line with bacterial microbiome investigations in IBS our human data set only provides an association between mycobiome dysbiosis and phenotype, not a causal link.[49] Whether the IBS patient mycobiome is capable of inducing visceral hypersensitivity may be addressed by future 'human to rat' fecal transfer experiments. So far, such an IBS-related colonization approach was only described in one publication where fecal suspensions of healthy volunteer and hypersensitive IBS patients were used to inoculate germ free Fischer 344 rats.[50] Rats with an IBS

microbiome showed significantly higher response to distension than those inoculated with healthy volunteer fecal suspension. Focus in this investigation was on the possible role of the bacterial microbiome. Post-colonization treatment with anti-fungals to reverse transfer-induced hypersensitivity was not performed but should be included in future experiments. Importantly however, successful colonization of 'normal' germ free rats with IBS mycobiota may not be enough to obtain proof of principle. Our own observations showed a role for fungi in maternal separated rats and possibly these fungi play a role in predisposed animals only. In relation to this, De Palma et al. investigated the gut microbiome in relation to altered behavior. Colonization of adult germ free maternal separated mice lead to anxiety-like behaviour and behavioural despair whereas colonization of germ free non-handled mice did not.[51] Possibly host factors present in maternal separated but absent in non-handled rats will also determine whether colonization with patient mycobiota leads to visceral hypersensitivity.

We showed that mycobiome dysbiosis and immune recognition of fungal antigens play an important role in visceral hypersensitivity in rat. Although we also found fungal dysbiosis in IBS patients there is no evidence for causality at this point. Proof of principle in human may be obtained by performing a well conducted clinical trial with fluconazole or nystatin. Due to increased resistance of pathogenic fungi, these classical fungicidals should probably not be used for regular IBS therapy but are best reserved for highly invasive and often lethal fungal infections. More subtle approaches of mycobiome modulation may however be suitable for IBS. This could include the use of probiotic fungal and bacterial strains able to induce a favorable mycobiome shift. In addition, our investigations with soluble β-glucans in rat suggest that targeting of fungal immune recognition instead of fungi is possible, even with a mild treatment protocol. For most optimal results in patients however, combined treatment with CRF receptor antagonists may be needed. From the present investigations we conclude that the gut mycobiome may be relevant for abdominal pain in IBS and should be addressed in future investigations.

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Figure legends

Figure 1. Differences in mycobiome between healthy volunteers (HV), hypersensitive irritable bowel syndrome (IBS-H) and normally sensitive IBS (IBS-N) patients. (A) Pie charts show distribution of the 30 most abundant species (+1 segment 'others') in HV, IBS-H and IBS-N respectively. (B) Shannon diversity index comparing fecal mycobiome of HV, IBS-H and IBS-N showing median and 25-to-75% and 2.5%-to-97.5% percentiles. N=16-20/group, **P<0.01, ***P<0.001 (Mann-Whitney). (C) Most robust biomarkers (in weight factors) resulting from stability selections conducted on annotated fungal ITS-1 gene sequences comparing IBS-H to HV. (D) Receiver operating characteristic (ROC) corresponding to stability selection, the computed area under the curve shows outstanding discriminating quality (AUC>0.9, p<0.01).

Figure 2. Fungi are important for water avoidance (WA)-induced visceral hypersensitivity of maternal separated (MS) rats. (A) Prevention of water avoidance-induced visceral hypersensitivity in maternal separated rats. Visceral sensitivity status is depicted by area under the curve of the relative response to colorectal distension (CRD). Prior to water avoidance, maternal separated and nonhandled (NH) rats received a 21 day treatment with vehicle alone, fluconazole or nystatin (n=8-9/group). (B) Schematic representation of fungal depletion and subsequent repopulation experiments (detailed in online methods). (C) Relative response to distension of three groups of maternal separated rats (n=8/group). After fluconazole/nystatin-mediated reversal of post-water avoidance visceral hypersensitivity, group 1 received pooled donor caecum content obtained from fluconazole/nystatin treated maternal separated rats. Group 2 received caecum content from non-treated maternal separated rats. Group 3 received caecum content from non-fluconazole/nystatin treated nonhandled rats. All data are mean +/- SEM, *P<0.05 (Wilcoxon signed rank).

Figure 3. Differences in mycobiome between nonhandled (NH) and maternal separated (MS) rats. (A) Visceral sensitivity status of rats used in the mycobiome evaluations depicted by area under the curve of the relative response to colorectal distension (data are mean ± SEM). (B)

Box and whiskers plot showing Shannon diversity index comparing fecal mycobiome of NH, MS and flucanosole/nystatin treated MS (MS-F/N) rats. The line inside boxes represents median, the width of the boxes and whiskers represents the 25-to-75% and 2.5-to-97.5% percentiles. (C) Left side dendrogram shows results of unsupervised cluster analysis based on Bray-Curtis dissimilarity. Right side; heat map of individual rats depicting relative abundance (%) of the 20 most abundant species. (D) Co-occurrence matrix of clustering rats. The more similar the mycobiome, the higher the tendency to cluster together. Co-occurrence values range from 0.0 (dark blue for rats who never cluster together) to 1.0 (dark red for rats who always cluster together). Rat numbers on axes are symmetric and represent individual rats. N=7-8, *P<0.05, **P<0.01 (Mann Whitney and Wilcoxon signed rank).

Figure 4. Mycobiome composition of rat feces and feed, and differences between selected fungal species. (A) Relative contribution species breakdown of the 20 most abundant fungal species in feed, nonhandled (NH), maternally separated (MS), and fluconazole/nystatin (F/N)-treated maternally separated rats. (B-E) Examples relative contribution of different species in rat feces (B) Monographiella nivalis is lower in maternally separated rats and further diminished in F/N treated maternally separated rats. (C-E) Acremonium zeae, Fusarium poae and uncultured Ascochyta all high in maternally separated and diminished upon F/N treatment. N=7-8, *P<0.05, **P<0.01, ***P<0.001 (Mann Whitney).

Figure 5. Fungal recognition via the Dectin-1/Syk signaling pathway is important in post water avoidance (WA)-stress visceral hypersensitivity. (A) Schematic representation of the experimental set-up and relative response to colorectal distension (CRD) after post-water avoidance treatment with vehicle alone or Syk inhibitor (1, 10 or 30 mg/kg). (B) Experimental set-up and relative response to colorectal distension of a peri-water avoidance, soluble β-glucan (SBG) administration protocol (three times gavage/24 hours of vehicle, 20 mg soluble β-glucan/kg or 50 mg soluble β-glucan/kg). (C) Experimental set-up and relative response to colorectal distension of a 1 week post- water avoidance, soluble β-glucan treatment protocol (vehicle alone or 50 mg soluble β-glucan /kg b.i.d.). Data are mean ± SEM. N=8-9/group, *P<0.05, **P<0.01 (Wilcoxon signed rank).

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Figure 6. Proposed mechanism of the role of fungi in stress-induced visceral hypersensitivity in maternal separated rats. Previously, a key role for mast cells and the histamine-1 receptor (H1R) was evidenced in the maternal separation model. Initial stress-induced mast cell degranulation and subsequent afferent activation and barrier dysfunction (phase 1) were shown to depend on peripheral Corticotrophin Releasing Factor (CRF). Our current investigations show that the prolonged post-stress pain response (phase 2) depends on the presence and recognition (via Dectin-1/Syk) of a unique mycobiome and related uptake of particulate β-glucans.

Table 1. Baseline characteristics of IBS patients and healthy volunteers used for fecal mycobiome analysis. a) Rectal sensitivity was assessed using an electronic rectal barostat procedure according to a standardized protocol. A VAS-score for pain≥20 mm at pressure step 26 mmHg was used as cut-off to define visceral hypersensitivity.48 b)Lactose-free diet; c)assessed on a 5-point Likert scale; *p<0.05, **p<0.01 ***p< 0.001 as tested by Chi2 for dichotomous and ANOVA for continuous variables; #) p<001 between IBS-H and IBS-N. ##) within 3 months prior to sample collection.

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Author names in bold designate shared co-first authorship.

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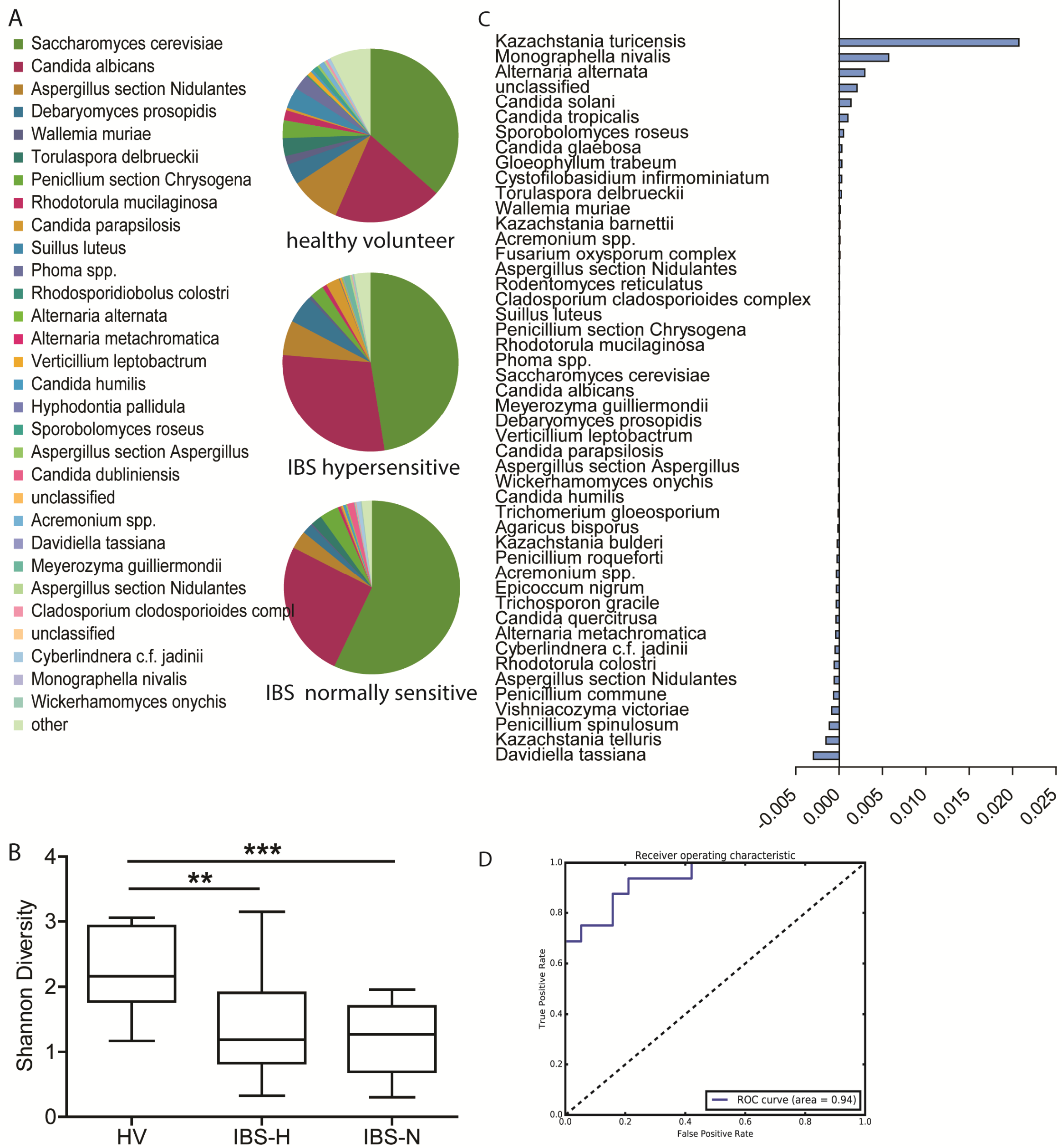
	Hypersensitive[a] IBS n=19	**Normosensitive IBS n=20**	**Healthy controls n=18**
Age (mean±SD years)	40.3±18.3	44.2±17.6	41.5±16.8
Gender (% females)	14 (73.7%)	14 (70.0%)	12 (66.7%)
IBS subtype*			
IBS-C	4 (21.1%)	5 (25.0%)	-
IBS-D	3 (15.8%)	10 (50.0%)	-
IBS-M	11 (57.9%)	3 (15.0%)	-
IBS-U	1 (5.3%)	2 (10.0%)	-
Current smoker (%)	5 (26.3%)	5 (20.0%)	1 (5.6%)
Alcohol use >14 units/week (%)	1 (5.3%)	1 (5.0%)	2 (11.1%)
BMI (mean±SD kg/m2)	22.7±3.5	25.4±4.5	23.8±4.6
HADS Depression-score (mean±SD)**	4.4±3.8	4.7±3.4	1.4±2.0
HADS Anxiety score (mean±SD)**	6.9±5.1	6.3±3.6	2.9±3.0
Medication use (%)			
*PPIs**	6 (31.6%)	5 (25.0%)	-
NSAIDs	2 (10.5%)	1 (5.0%)	-
*SSRIs**	6 (31.6%)	1 (5.0%)	1 (5.5%)
*Motility altering drugs***	7 (36.8%)	2 (10.0%)	-
Antibiotics/antifungal (%)##	-	-	-
Diet (%)	1 (5.3%)[b]	-	-
Pro/prebiotics (%)	-	1 (5.0%)	1 (5.6%)
Symptom score (mean±SD of 14-day diary)[c]			
*Abdominal pain****	2.6±0.8	2.2±0.8	1.1±0.2
*Abdominal discomfort****	2.8±0.8	2.3±0.7	1.1±0.2
*Bloating****	2.5±0.8	2.1±1.0	1.2±0.2
*Belching***/#	1.8±0.7	1.5±0.6	1.1±0.3
*Nausea***/#	2.1±1.2	1.8±1.0	1.0±0.0
*Flatulence****	2.5±0.9	2.5±1.2	1.4±0.4
*Constipation***/#	1.8±0.7	1.4±0.5	1.1±0.2
*Diarrhea***/#	1.6±0.7	1.3±0.3	1.1±0.1
*Overall symptom burden****	2.8±0.7	2.3±0.7	1.1±0.2
Pressure step for first VAS score pain > 10mm (mean±SD mmHg)***/#	13.0±8.7	37.6±10.9	42.3±9.0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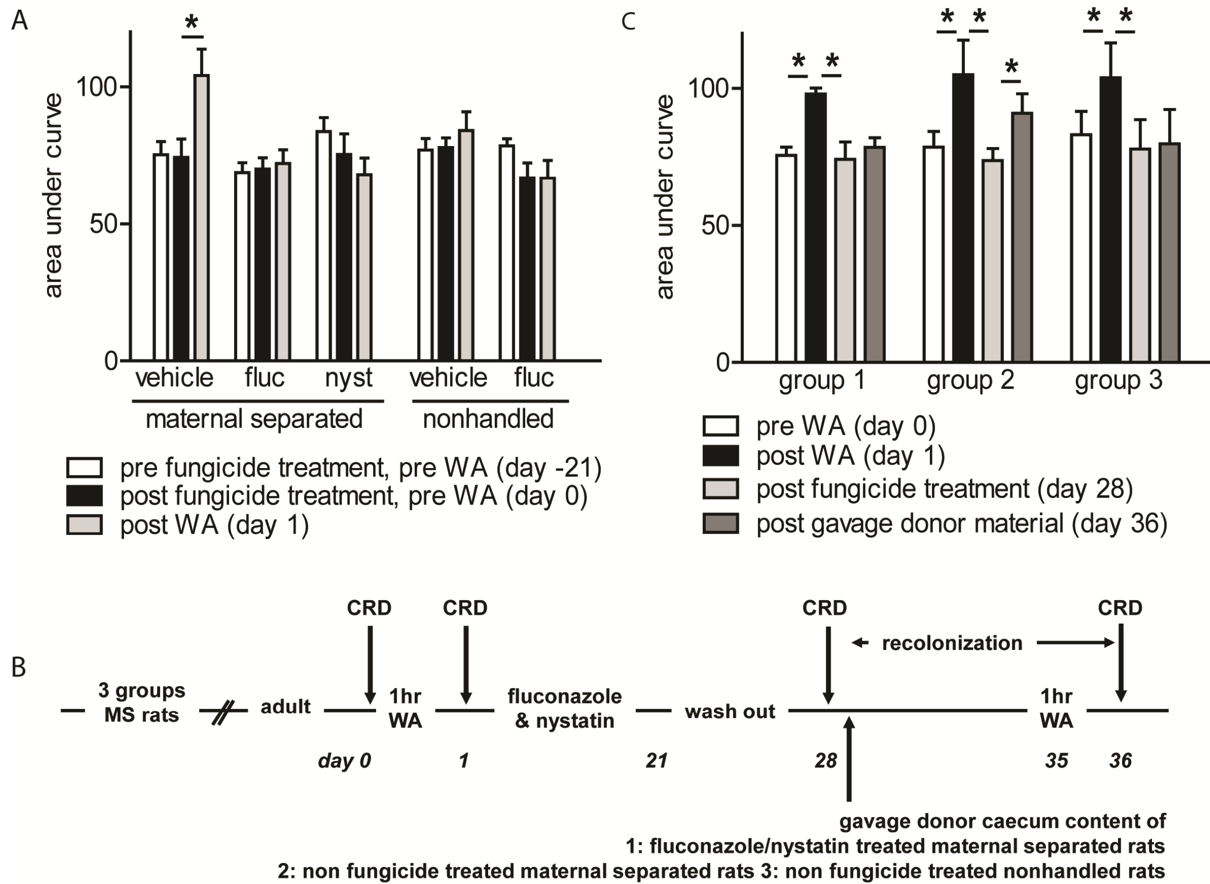

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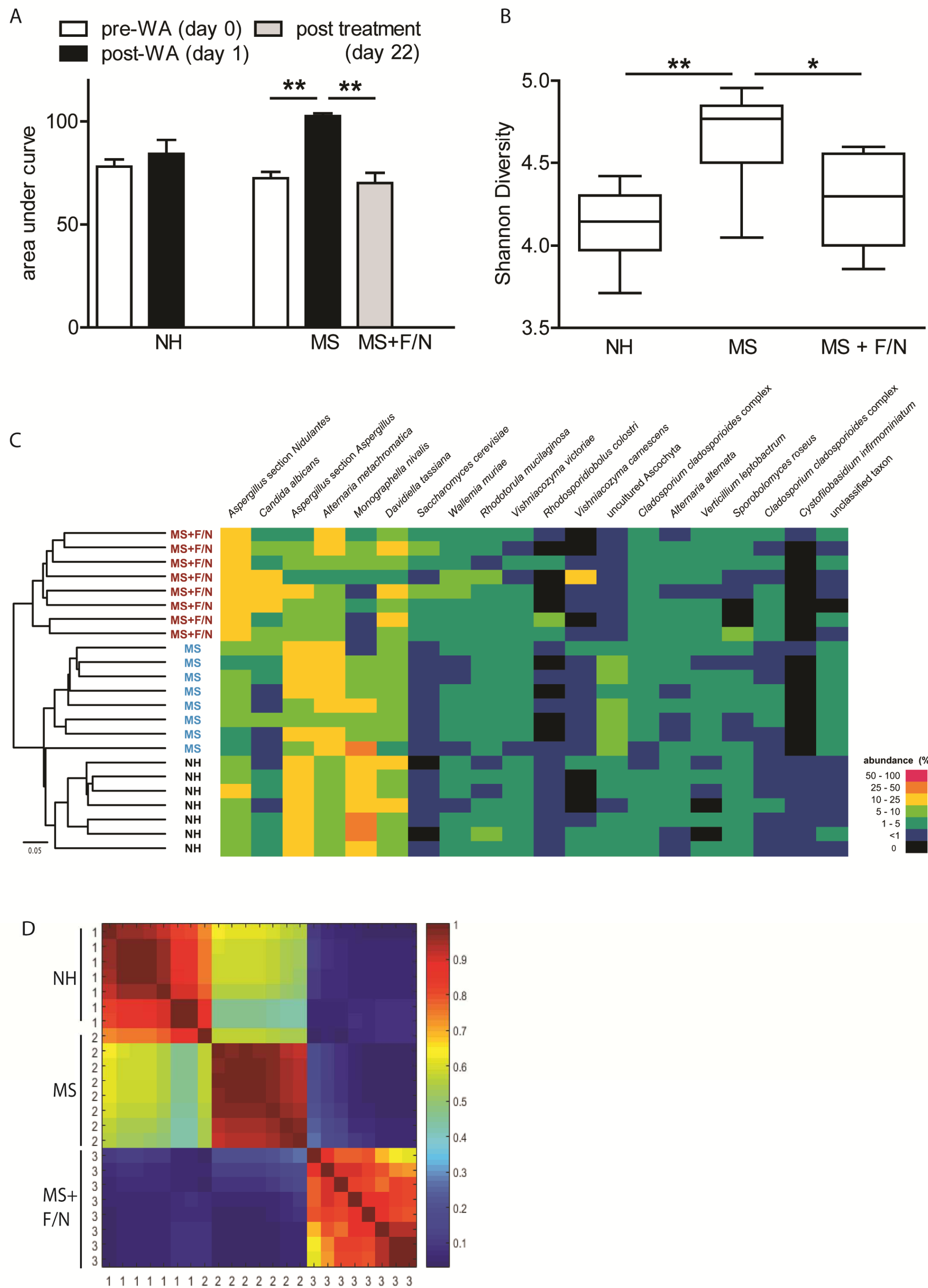

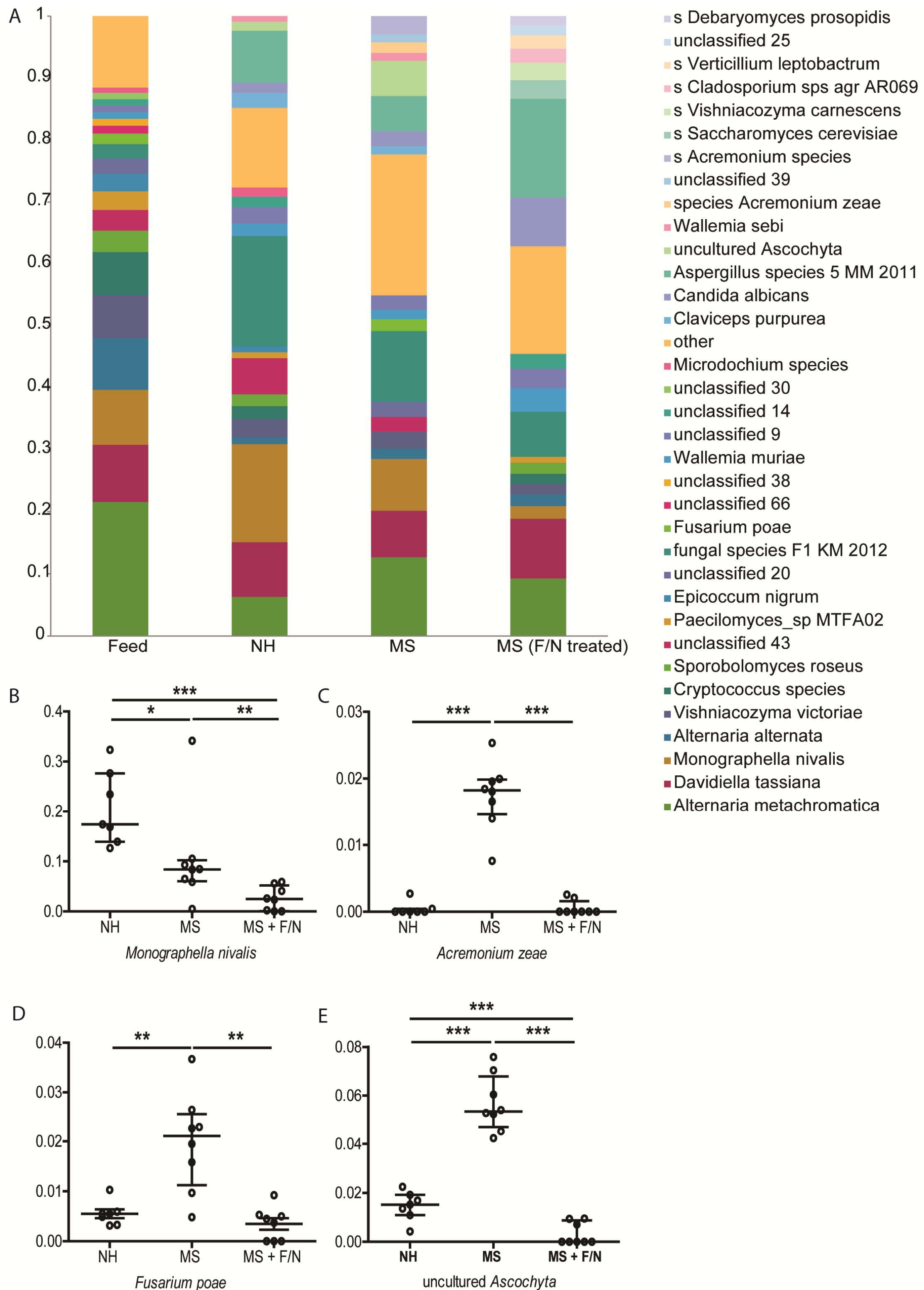

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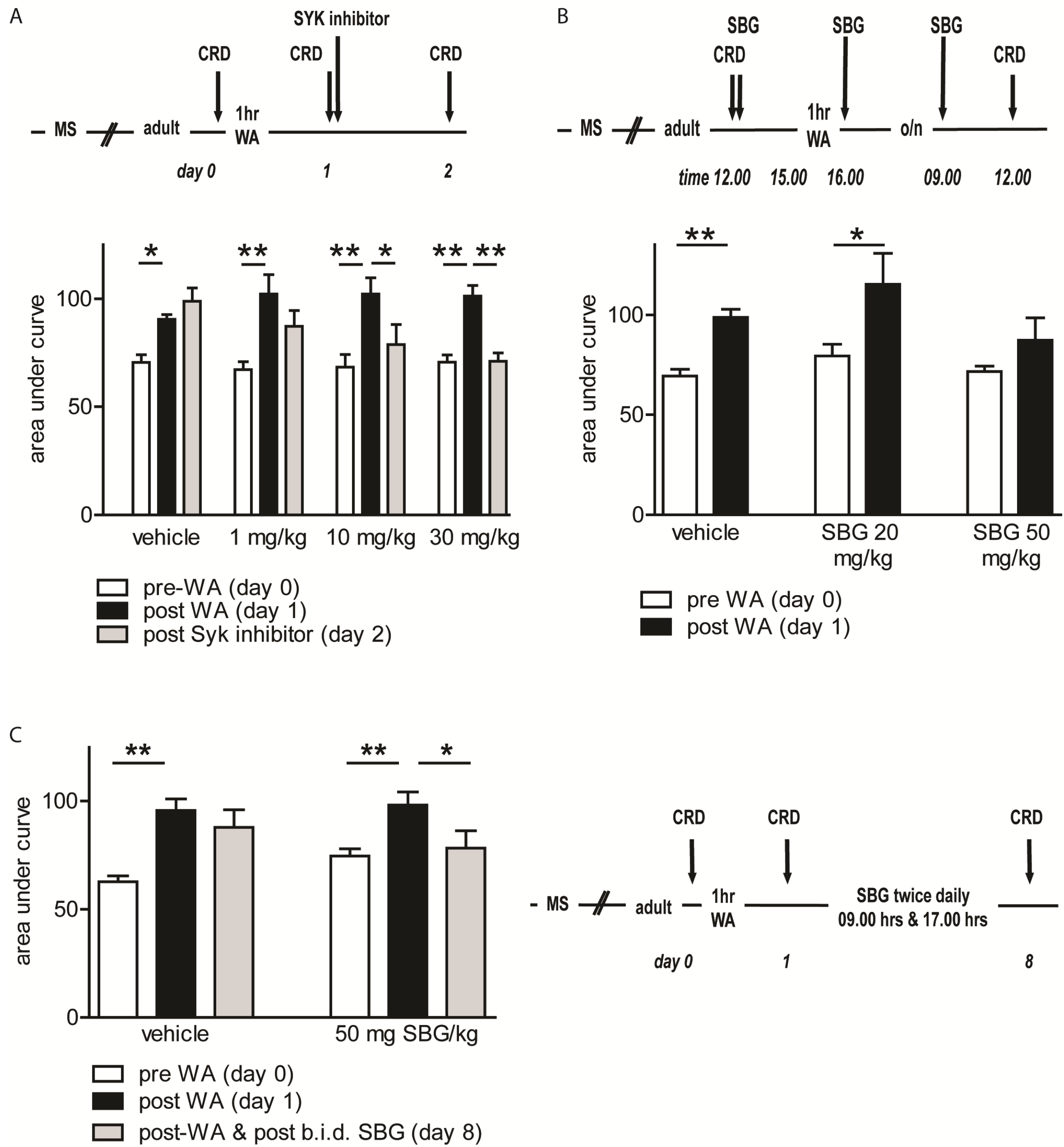
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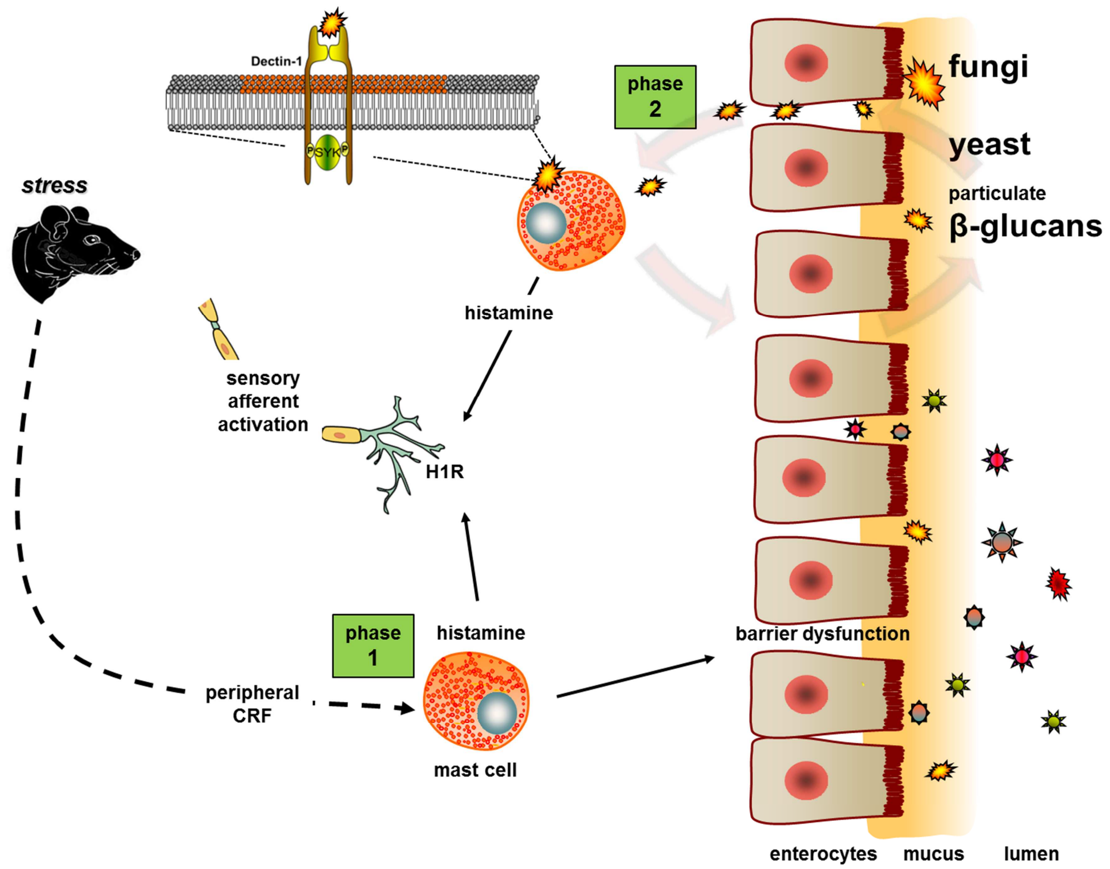

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Supporting documents to:

Intestinal Fungal Dysbiosis Associates With Visceral Hypersensitivity in Patients With Irritable Bowel Syndrome and Rats

This document includes:

- Material and Methods

-Supplementary figures 1-15

-Supplementary tables 1 & 2

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MATERIAL AND METHODS

Maternal Separation

Neonatal maternal separation in Long Evans rats predisposes for stress-induced visceral hypersensitivity at adult age.[1, 2] This model mimics early adverse life events shown to be associated with increased risks of IBS later in life.[3] Maternal separation was carried out as follows; on postnatal day 2, female pups were removed from the litter and dams were separated from remaining pups 3 hours daily for a 12 day period (postnatal day 2 to 14) by placing the dams in another room. During separation the cage with litter was placed on a heating mat to maintain pup body temperature. Pups were weaned at day 22. Non-handled pups were nursed normally.

Colonic distension protocol and water avoidance (WA)

Distensions were performed at the minimum age of 4 months with a latex balloon (Ultracover 8F, International Medical Products BV, Zutphen, The Netherlands) and carried out as described previously.[2] The balloon catheter was inserted under short isoflurane anesthesia and, after a 20 min recovery period colonic distension was achieved by inflation of graded volumes of water (1.0, 1.5, and 2.0 mL) into the balloon. Length and diameter of the balloon during a 2 mL maximum volume distension were 18 and 15 mm, respectively. After each 20 sec distension period the volume of water was quickly removed and an 80 sec resting period was introduced. For acute stress at adult age (i.e. WA), rats were positioned on a pedestal (8 · 8 · 10 cm) attached to the bottom of a plexiglass tank (25 · 25 · 45 cm). The tank was filled with fresh tap water at room temperature (21 ºC) within 1 cm of the top of the pedestal and rats remained in the tank for 1 hour.

Measurement of the visceromotor response to colonic distension and data analysis

Visceral hypersensitivity in patients is assessed by rectal distensions: hypersensitive IBS patients perceive pain during luminal distensions at lower volumes or pressures than normal controls. Pain scores are often evaluated by self-rating questionnaire (i.e. visual analogue score) that cannot be assessed in rats. However, colorectal distension in rat leads to reproducible contractions of abdominal musculature; the so called visceromotor response and the quantification of these contractions by electromyography (EMG) is often used to assess visceral pain.[4] In short, a telemetric transmitter was sutured in the abdominal cavity to record EMG signals from two connected electrodes placed in the abdominal muscles as described earlier.[2] During distensions, animals were placed in a standard macralon cage that was positioned on top of an equally sized flat receiver (DSI). The receiver was linked to a Biopac MP100 data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA) and a personal computer via a raw data analog converter (Data Sciences International). Data were acquired with AcqKnowledge software (Biopac Systems Inc.) and then analyzed. Briefly, each 20 sec distension period and its preceding 20 sec of baseline recording were extracted from the original raw EMG data file. After correction for movement and breathing, data were rectified and integrated. Absolute datasets were then obtained by subtracting the 20 sec baseline recording from the 20 sec distension result. Similar to our previous publications final results were evaluated from normalized data sets, which were calculated from the absolute data by setting the 2 mL value of the first distension at 100%. The area under the curve of these relative responses was calculated for individual rats and used for statistical analyses by Wilcoxon-signed ranks test

(performed with SPSS for Windows, version 16.0; SPSS Inc., Chicago, IL, USA). Corresponding per volume line diagrams are given in Supplementary Figures 9-15.

In vivo experiments

Pre-water avoidance anti-fungal treatment. The drinking water of adult rats was supplemented with fluconazole (0.5 mg/ml), nystatin (200 U/ml) or vehicle alone (i.e. demineralized water) for 3 consecutive weeks. Just prior and directly after anti-fungal treatment (day -21 and day 0 respectively), the response to distension was assessed to exclude direct fungicide induced effects on baseline (pre-WA) sensitivity to distension. Directly after the second distension protocol rats were subjected to WA (day 0) and a third round of colorectal distensions on day 1.

Post-water avoidance anti-fungal treatment and subsequent fungal repopulation (schematic representation in Figure 2B). The response to distension was assessed pre- and 24 hours post-WA in 3 groups of MS rats. Next, all groups were treated with anti-fungals (combination treatment; 0.25 mg fluconazole/ml & 200 U nystatin/ml) in drinking water for 3 consecutive weeks and then transferred (day 21) to individually ventilated cages until the end of the experiment. During the last 3 days of a 7 days fluconazole/nystatin washout period all groups received omeprazole (25 mg/kg daily). Post fungicide-treatment response to distension was then assessed at day 28. Directly after this, rats were gavaged with 1 ml fresh caecum content that was pooled from N=4-5 donor rats, pushed through a stainless steel wire mesh filter and diluted 1:1 with water. Prior to being used as donor in these experiments all donor rats were evaluated for pre- and post-WA sensitivity status (for results see Supplementary Figure 2). In recipient rats, group 1 received caecum content of MS donor rats that were treated with fluconazole/nystatin for three weeks. Recipient group 2 received caecum content of non-fungicide treated MS donor rats and group 3 received pooled content of non-fungicide treated NH donors. All 3 recipient groups were then subjected to a second WA (at day 35) and a final distension protocol at day 36.

Post-water avoidance treatment with Syk inhibitor (schematic representation in Figure 5A). The response to distension was assessed in 4 groups of MS rats pre- and 24 hours post-WA. Directly after WA, rats received 1 oral gavage with vehicle alone (1% methylcellulose solution in water; group 1) or Syk inhibitor (GSK143, kind gift from GSK, Stevenage, UK; 1, 10 or 30 mg/kg; groups 2, 3 and 4 respectively)[5] and were subjected to a final distension protocol 24 hours post WA.

Peri-water avoidance treatment with soluble β-glucans (schematic representation in Figure 5B). Directly after recording the first response to distension at 12.00 hrs, 3 groups of MS rats were gavaged with either vehicle alone (demineralized water) or soluble β-glucan (SBG, kind gift from Biotech Pharmacon ASA, Tromsø, Norway; 20 or 50 mg/kg). 3 hours later rats were subjected to WA, and gavaged again directly after. The final gavage was given the next morning at 09.00 hrs and response to distension was assessed at 12.00 hrs.

Post-water avoidance treatment with soluble β-glucan (schematic representation in Figure 5C). The response to distension was assessed pre-WA (day 0) and 24 hrs post WA (day 1) in 2 groups of MS rats. During the next 7 days rats were gavaged twice daily (at 09.00 and 17.00 hrs) with vehicle alone or 50 mg soluble β-glucan /kg. On day 8, response to distension was again assessed in both groups of rats.

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Fecal pellet/stool collection

Human stool samples were collected at home, brought to the lab and frozen within 24 hours after defecation. Rat fecal pellets were collected directly from the anus just prior to distension protocols. All samples were stored in -80 ^{0}C until use.

DNA extraction

Rat and human fecal samples were resuspened in Tris-EDTA buffer (pH7.5) containing 1.2 mM sorbitol, 250 U/ml lyticase and 0.2% β-mercaptoethanol. After 1 hour incubation at 37ºC, pellets were spun down and taken up in Tris-EDTA lysis buffer with 2% T-X-100 and 8M guanidine thiocyanate. This mix was incubated at room temperature for 4 hours, spun down and incubated with silica suspension to complex liberated DNA. Upon centrifugation complexes were washed with guanidine thiocyanate buffer (twice), 70% ethanol (twice) and acetone (twice), dried and eluted in aqueous low-salt buffer. Finally, cetyltrimethylammonium bromide (2% CTAB, 20 minutes, 65 ºC) incubation and subsequent chloroform/iso-amyl alcohol extraction were used for final DNA purification. Remaining DNA was precipitated by isopropanol/sodium acetate, washed and stored in TE buffer until use.

Sequencing of fungal ITS amplicons

Barcoded fungal internal transcribed spacer regions (ITS) amplicons were generated using a two-step PCR approach. Fungal ITS 1 regions were first amplified with the following primers: forward 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and reverse 5′-GCTGCGTTCTTCATCGATGC-3.[6] Each reaction contained 300 times diluted and purified fecal DNA, hot start PCR master mix (Thermo Scientific) and nuclease free PCR grade water to a 50 µl final reaction volume. PCR reactions consisted of an initial denaturation step of 95 °C, for 5 min and 30 amplification cycles (95 °C for 30 sec), annealing (52 °C for 45 sec) and elongation (72 °C for 1 min) and final extension step (72 °C for 10 min) followed by cool down (10 min at 4 °C). A negative control (blank) was included for each 24 PCR reactions. Next a second set of primers was used to generate fungal ITS-1 fragments that included overhanging adapter sequences for compatibility with Nextera XT tagmentation: next-ITS-BITS-F: TCGTCGGCAGCGTCACCTGCGGARGGATCA and nex-ITS-B58S3-R GTCTCGTGGGCTCGGGAGATCCRTTGYTRAAAGTT (adapted from Bokulich & Mills[7]). 5 µl of the previous PCR products were amplified for 10 cycles with an annealing temperature 49 °C. Reactions were cleaned by solid-phase reversible immobilization (SPRI) using AMPure XP SPRI beads (Beckman Coulter, Inc.). Dual barcodes (8 bp) and Illumina sequencing adapters were attached using the Nextera XT Index Kit (Illumina, San Diego, CA) according to manufacturer's protocols. Barcoded amplicons were quantified using the Caliper LabChip GX II system (Perkin Elmer, Hopkinton, USA), normalized to the same concentrations, pooled, and gel purified using the Qiaquick spin kit (Qiagen). Pooled amplicons were 250-bp paired-end sequenced using the MiSeq system (Illumina).

Raw Illumina fastq files were demultiplexed, quality filtered, and analyzed using modules implemented in the Mothur software platform.[8] > 35.10^6 raw reads were merged into 8,785,442 paired reads. Rigorous quality filtering resulted in 7,694,074 unique paired reads with a mean length of 154 nt. Unique sequences were taxonomically classified by the RDP-II Naïve Bayesian Classifier[9] using a 60% confidence threshold against the Mothur formated UNITE Database (Version No. 7).[10] Distances between ITS library compositions were visualized in UPGMA

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dendrograms showing Bray-Curtis statistics. The raw sequence data generated in this study will be deposited in the European Nucleotide Archive upon acceptance of the manuscript.

Sequencing of bacterial 16S amplicons

Analysis of the microbiome composition was performed by massive sequencing of the V4 hypervariable region of the 16s rRNA gene on the illumina MiSeq sequencer. Barcoded DNA fragments spanning the Archaeal and Bacterial V4 hypervariable region were amplified with a standardizing level of template DNA (100pg) to prevent over-amplification. These amplicons, generated using adapted primers 533F and 806R, were bidirectionally sequenced using the MiSeq system (Illumina, San Diego, CA) as described previously.[11]

All data extraction, pre-processing, analysis of OTUs and classifications were performed using modules implemented in the Mothur software platform as in Kelder *et al.*[12] except where noted below. A total of 3120791 high-quality sequences were aligned using the 'align.seqs' command and the Mothur-compatible Bacterial SILVA SEED database. A total of 6897 unique sequences were retrieved using this pipeline. Sequences were clustered in 1415 OTUs using average linkage clustering and a 97% sequence-identity threshold. Sequences were taxonomically classified by the RDP-II Naive Bayesian Classifier using a 60% confidence threshold. Community profiles were compared by Bray–Curtis dissimilarity of OTU abundance. Sequences were normalized to 1000 sequences per samples (subsampling method).

Statistical analysis

Fungal biomarker species that allow accurate discrimination among groups were selected by means of the elastic net algorithm.[13] Elastic net method is naturally applicable to structured and high-dimensional datasets. It is a regularized method, that combines the advantages of two techniques: LASSO[14] (sparsity, retaining the variable selection property of reducing coefficients to exact zero values provided by LASSO) and ridge regression (smoothness, tendency of shrinking coefficients to small values for correlated trending towards each other). This combination allows for the selection of the most important biomarkers, while taking the correlation (so called 'grouping effect') among them into account.

Our statistical analysis is based on application of the adapted version of the elastic net algorithm (with Hinge loss function) which is specifically tailored for discriminating fungal species in the collected dataset. We train the model by taking the gradient of the loss that is estimated at each sample at a time (stochastic gradient descent learning). It consists of learning and stability selection modules and allows for robust and reliable identification of the biomarkers. Formally, our method is able to shrink coefficients to zero by imposing an L1-penalty on the classification coefficients; doing so allows for an easily interpretable model by viewing any nonzero coefficients as the predictors that have the strongest predictive power. We note that the elastic net algorithm allows building multivariate models, and thus, can lead to the identification of a group of fungal biomarkers that jointly have an effect on a differentiation among healthy volunteers vs normally sensitive IBS, heathy volunteers vs hypersensitive IBS, normal sensitive IBS vs hyper sensitive IBS patients. While the biomarkers identified by elastic net algorithm usually lead to statistically significant results, they can frequently be unstable. In our approach, we address this problem via stability selection procedure coupled with the model selection. Biomarker stability is reflected in the frequency that a particular biomarker was identified in multiple simulations on a re-randomized dataset. This measure is especially relevant for small to

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medium sized data collections, as in this study where the number of patients diagnosed IBS with is limited.

The performance measure used for a binary classification task is a Receiver-Operating-Characteristics Area-Under-Curve (ROC AUC). The ROC can be understood as a plot of the probability of correctly classifying healthy volunteers vs normally sensitive IBS or heathy volunteers vs hypersensitive IBS or normal sensitive IBS vs hyper sensitive IBS patients. Thus, the AUC score of an ROC curve can be considered a measure of the predictive accuracy of the model.

To avoid over-fitting, we used a 10-fold stratified cross-validation procedure over the training partition of the data (80%) while the remaining 20% was used as the testing dataset. Parameters to be selected are ratio between L1, L2 norms, and regularization parameter. Stability selection was performed by randomly subsampling 80% of the data 100 times. During stability selection procedure, all features having non-zero weighting coefficient were counted. These counts were normalized and converted to stability coefficients having value between 1.0 for the feature which was always selected and 0.0 for feature which was never selected. We used Python (version 2.7.8, packages Numpy, Scipy) for implementing elastic net model and the R version 3.1.2 for visualization.

To analyse the fungal composition of humans/rats, an unsupervised co-regularized spectral clustering algorithm was applied to the dataset.[15, 16] In short, this multi-view clustering algorithm allows for identification of clusters comprised of humans/rats with similar fungal/microbial profiles in an unbiased and robust manner. The method stems from a recently proposed class of multi-view clustering algorithms[17] 16 that have been reported to notably outperform standard techniques (e.g. k-means, hierarchical clustering, etc.) in clustering accuracy and stability. Multi-view algorithms (closely related to cluster ensembles and consensus techniques) aim to combine multiple clustering hypotheses for increased accuracy and are not limited to a single similarity measure, thus leading to robust and reliable results.

Whole mount staining mesenteric windows.

Mouse mesenteric windows were carefully preserved and pinned down in a Sylgard dish (see Supplementary Figure 8A). After 30 minutes pre-incubation with 3% BSA in PBS, mesenteric windows were incubated with beta glucan (5 mg/ml PBS, Biotec Pharmacon) or PBS-BSA for 30 minutes and washed. Subsequently, windows were exposed to Texas-red labeled avidin (30 minutes), washed, carefully removed from Sylgard dish and mounted in DAPI containg Vectashield mounting medium. In non-fixed tissues, avidin interacts with mast cell heparin of degranulating mast cells only.[18]

Dectin-1 expression on HMC-1 cells

The human mast cell line HMC-1 was kindly provided by Dr J.H. Butterfield (Mayo Clinic, Rochester, MN). Dectin-1 surface marker expression was analyzed with mouse anti Human Dectin-1 antibody conjugated to Alexa Fluor 647 (Serotec, Raleigh, US). Before staining, cells were pretreated with human Fc-receptor blocking reagent (Miltenyi Biotec, Leiden, The Netherlands). Data was acquired on an LSRFortessa analyzer (BD Biosciences, Breda, The Netherlands) and analyzed with FlowJo software (Treestar, San Carlos, US).

HPLC detection of histamine in HMC-1 cell supernatants.

HMC-1 cells (10^6/ml) were incubated at 37°C for 10 min in HBSS with 50 µg/ml compound 48/80 or heat-inactivated *Candida albicans* (ATCC 18804, $2.5 \cdot 10^6$/ml). Vehicle treated cells served as negative control. After stimulation, cells were spun down and norvalin (50 nM) was added to supernatants as internal standard. Similarly, histamine standard solutions were prepared by mixing 1 µM histamine with 50 nM norvaline in acetonitrile. Before detection by HPLC, cell culture supernatants were deproteinized by acetonitrile treatment. HPLC detection was performed with a Dionex Ultimate 3000 HPLC equipped with a RF 2000 fluorimetric detector (Dionex, Germering, Germany). Before injection, histamine was derivatized with o-phthalaldehyde (OPA) in autosampler and injected automatically onto a BDS hypersil C18 column (Thermo Scientific, Rockford, IL, USA) (150x4.6 mm, particle size 3 µm, 2 columns in tandem) equipped with a guard cartridge C 18 ODS 4x3 mm (Phenomenex, Utrecht, The Netherlands). Gradient elution was performed with the following solvents; A, sodium phosphate buffer (125 mM, pH 7), water (20:80) containing 0.5% of tetrahydrofurane; B, sodium phosphate buffer (125 mM, pH 7), tetrahydrofurane, acetonitrile, water (5:7:40:48). Gradient conditions were: 13 min (75% B), 8 min isocratic, 5 min (0% B) and elution flow was 0.7 ml/min. Detection wavelengths: excitation: 345 nm, emission: 485 nm. Under these conditions, the retention time for histamine derivative is 16.5 minutes. Results are expressed as histamine/norvaline ratio.

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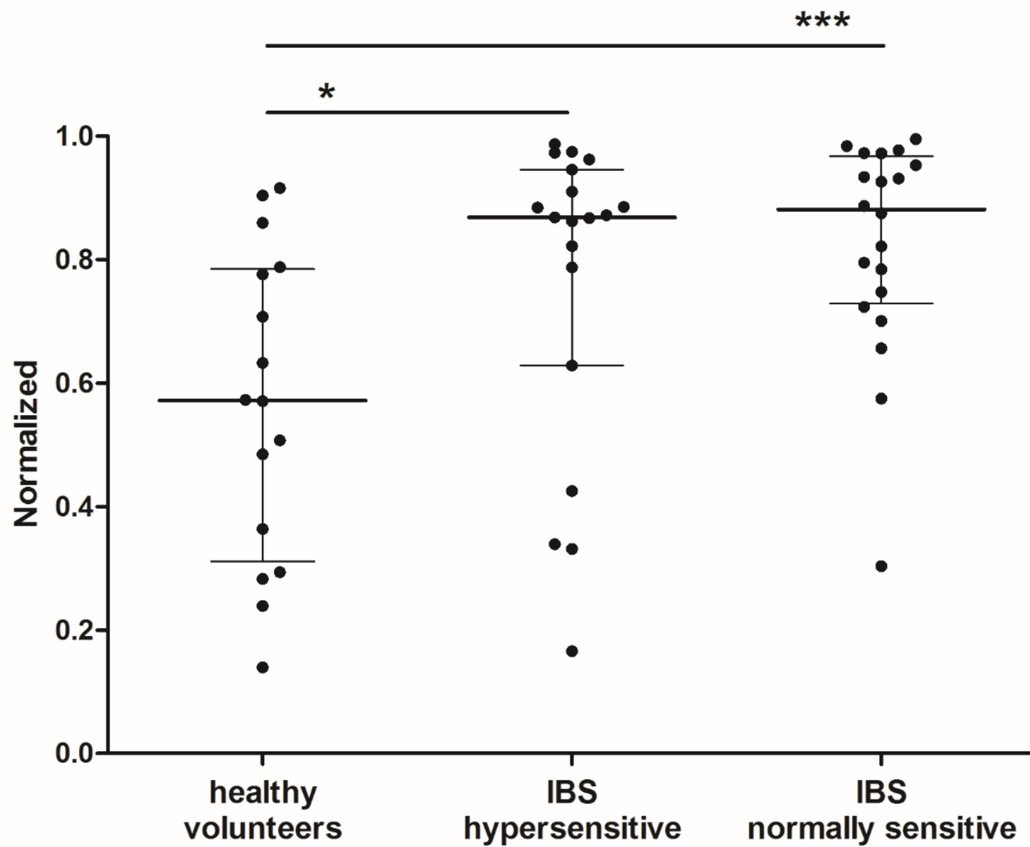
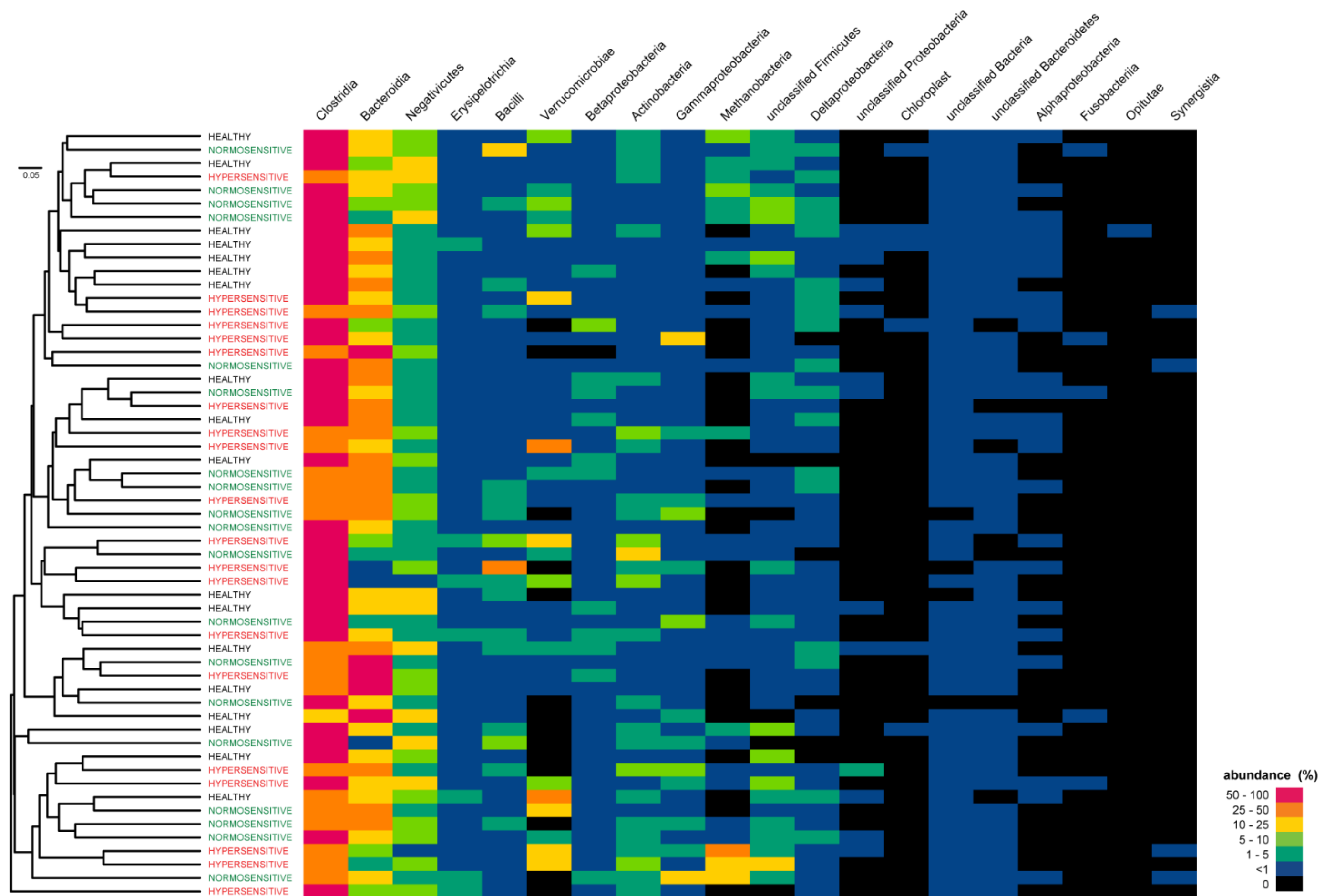


Supplementary Figure 1. Combined relative presence of Saccharomyces cerevisiae & Candida albicans. Intergroup differences between healthy volunteers, hypersensitive IBS and normally sensitive IBS. Data are mean ± SEM. *$P<0.05$, ***$P<0.001$ (Wilcoxon signed rank).



Supplementary Figure 2. No difference in bacterial microbiome between healthy volunteers, normosensitive IBS and hypersensitive IBS. Left side dendrogram shows the result of Bray-Curtis dissimilarity. Right side heatmap shows relative abundance of bacterial classes detected in individual fecal samples.

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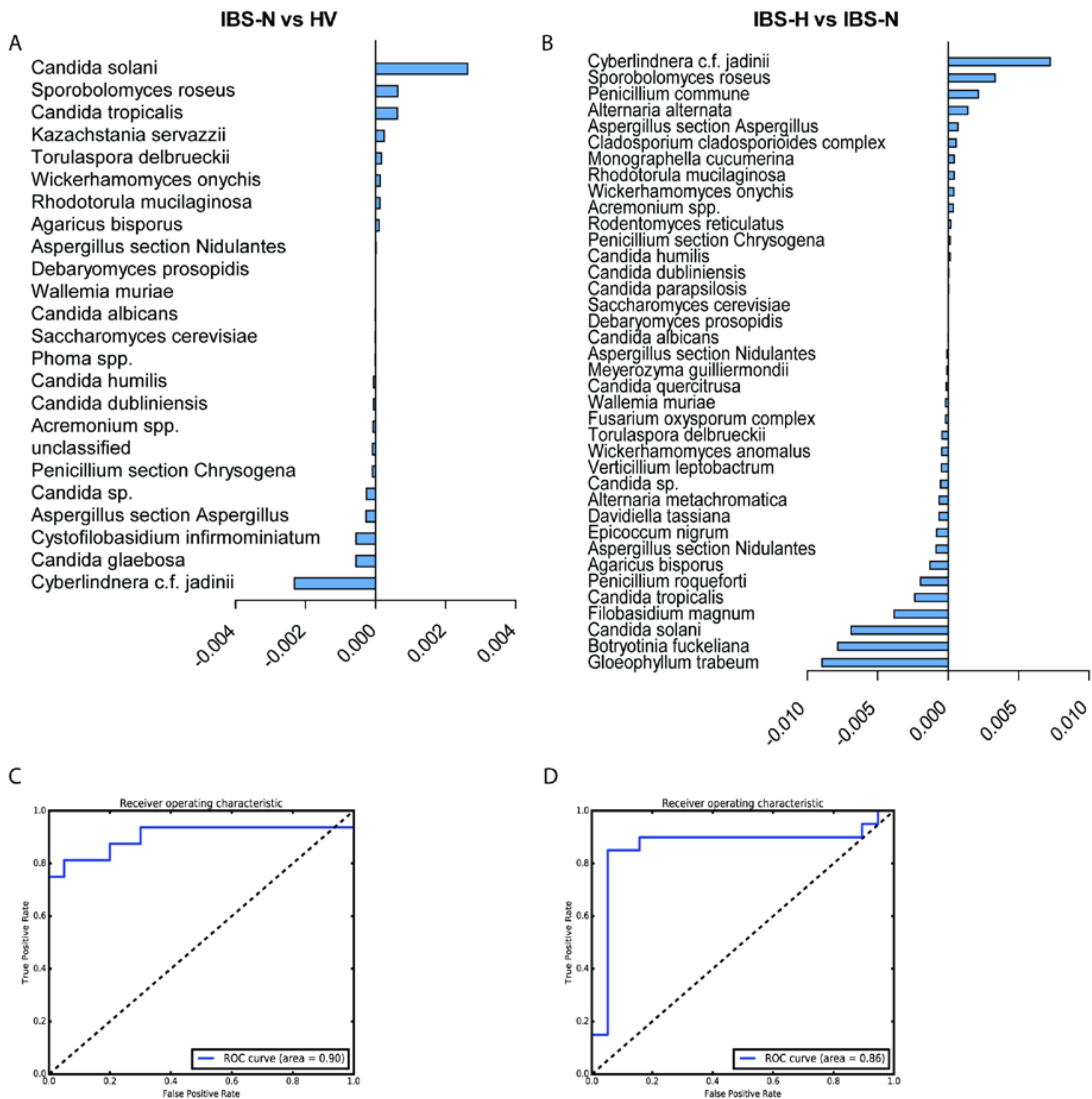
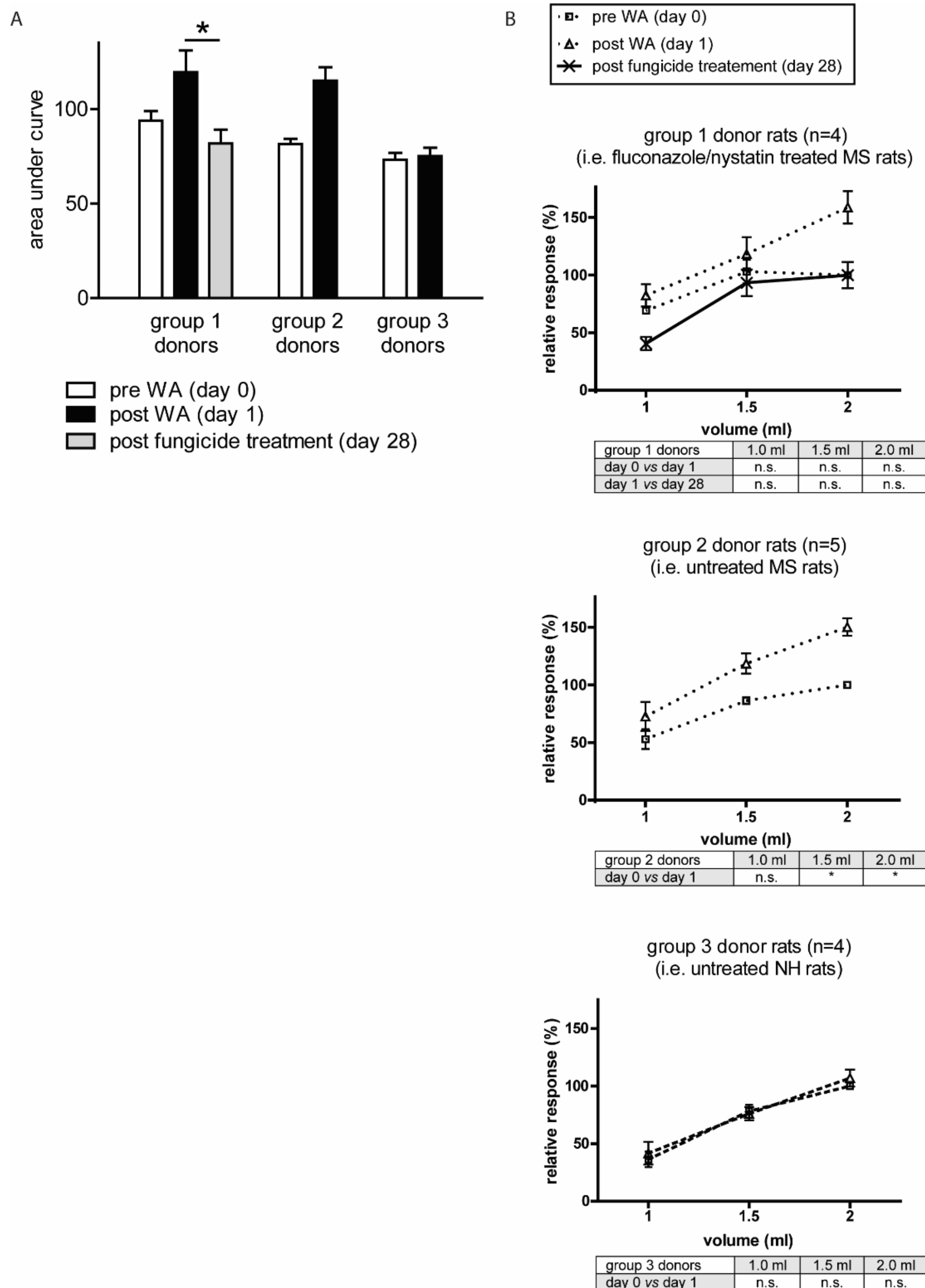
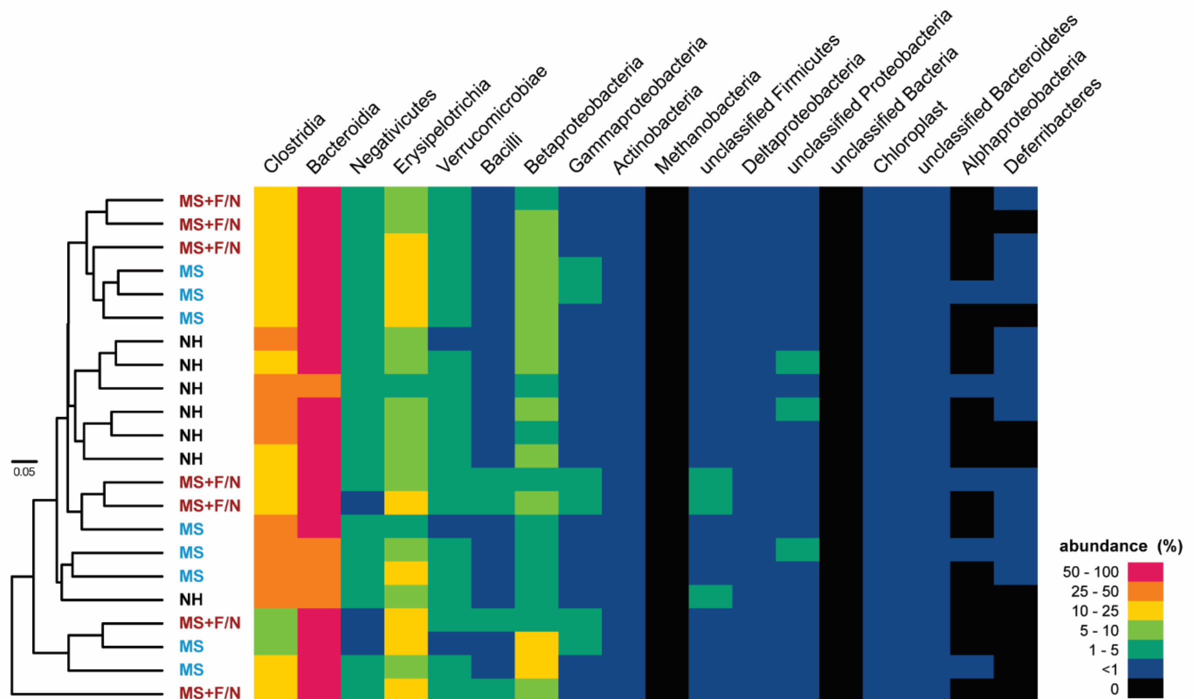


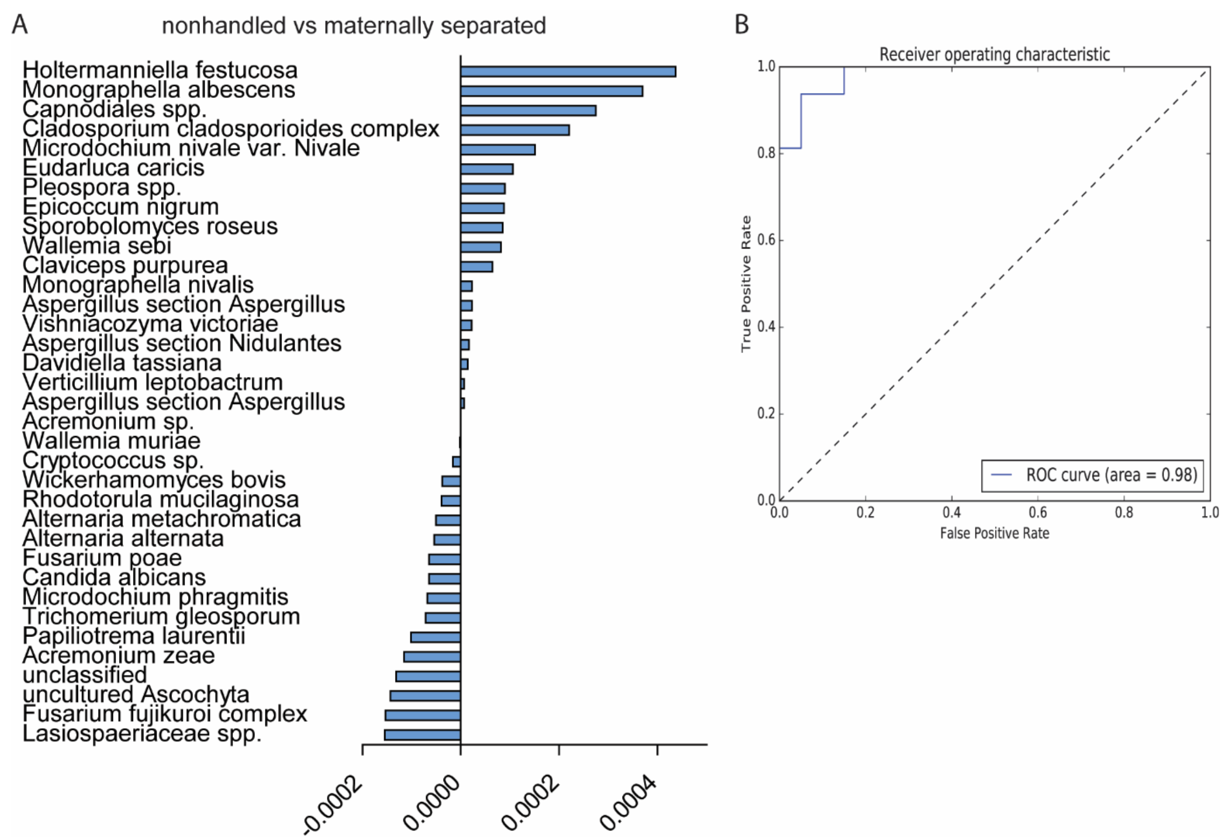
Supplementary Figure 3. Fecal human mycobiome signatures identified by stability selection. (A) Intergroup differences between normally sensitive IBS (IBS-N) and healthy volunteers (HV) and **(B)** between hypersensitive IBS (IBS-H) and IBS-N. The respective receiver operating characteristic (ROC) curves are depicted in **(C)** and **(D)**. The area under the curves (AUC; 0.90 and 0.86 respectively, $p<0.01$ in both simulations) show good (AUC 0.8-0.9) discriminating quality.



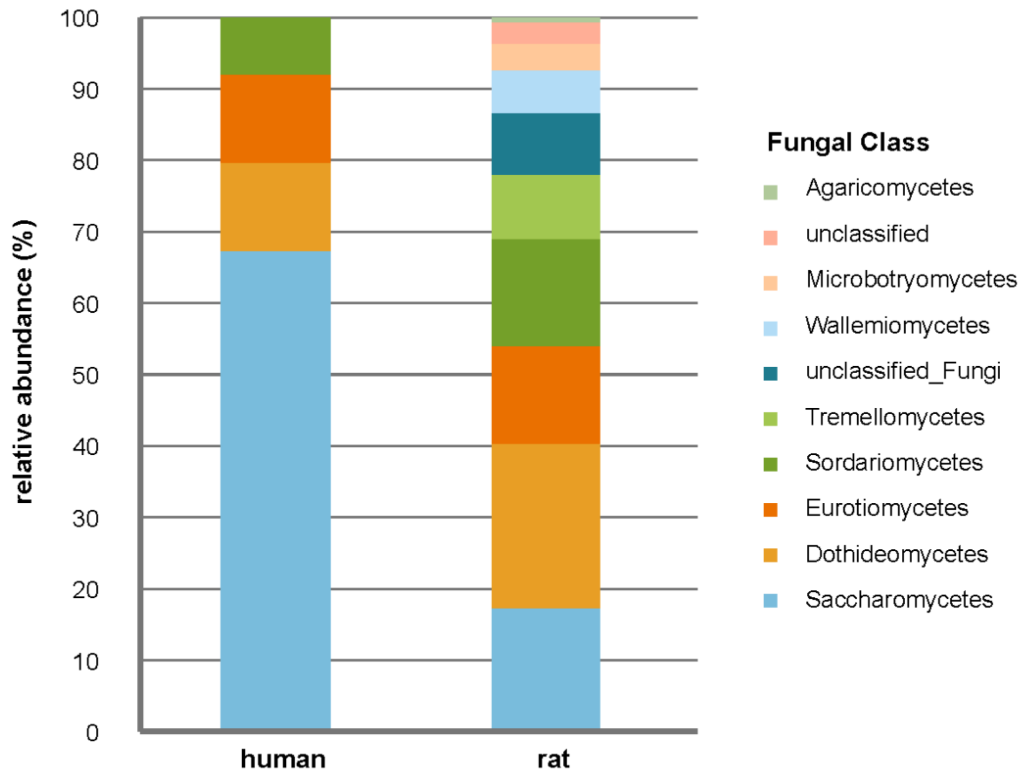
Supplementary Figure 4. Relative response to distension of donor rats (n=4-5) used in fecal transplantation experiments (results of transplantation experiments are shown in Fig 2C). Caecum content was gathered after the last distension protocol in each group. Group 1 donors: maternally separated rats treated with fluconazole/nystatin. Group 2 donors: non fungicide treated maternally separated rats. Group 3 donors: non fungicide treated non-handled rats. **(A)** Visceral sensitivity status is depicted by area under the curve of the relative response to colorectal distension. **(B)** The same results as volume/relative response line diagrams. All data are mean ± SEM, n.s. not significant, *P<0.05.



Supplementary Figure 5. No difference in bacterial microbiome composition between non-handled (NH), maternal separated (MS) and post fluconazole/nystatin maternal separated (MS+F/N) rat groups. Left side dendrogram shows the result of Bray-Curtis dissimilarity. Right side heatmap shows relative abundance of bacterial classes detected in individual fecal samples.



Supplementary Figure 6. Fecal rat mycobiome signature identified by stability selection. (A) Intergroup differences between nonhandled (NH) rats and maternally separated (MS) rats. The corresponding receiver operating characteristic (ROC) curve is depicted in **(B)**. The area under the curve (AUC; 0.98) indicated outstanding (AUC>0.9) discriminating quality.



Supplementary Figure 7. Fecal mycobiome composition. Relative contribution class breakdown in human and rat feces.

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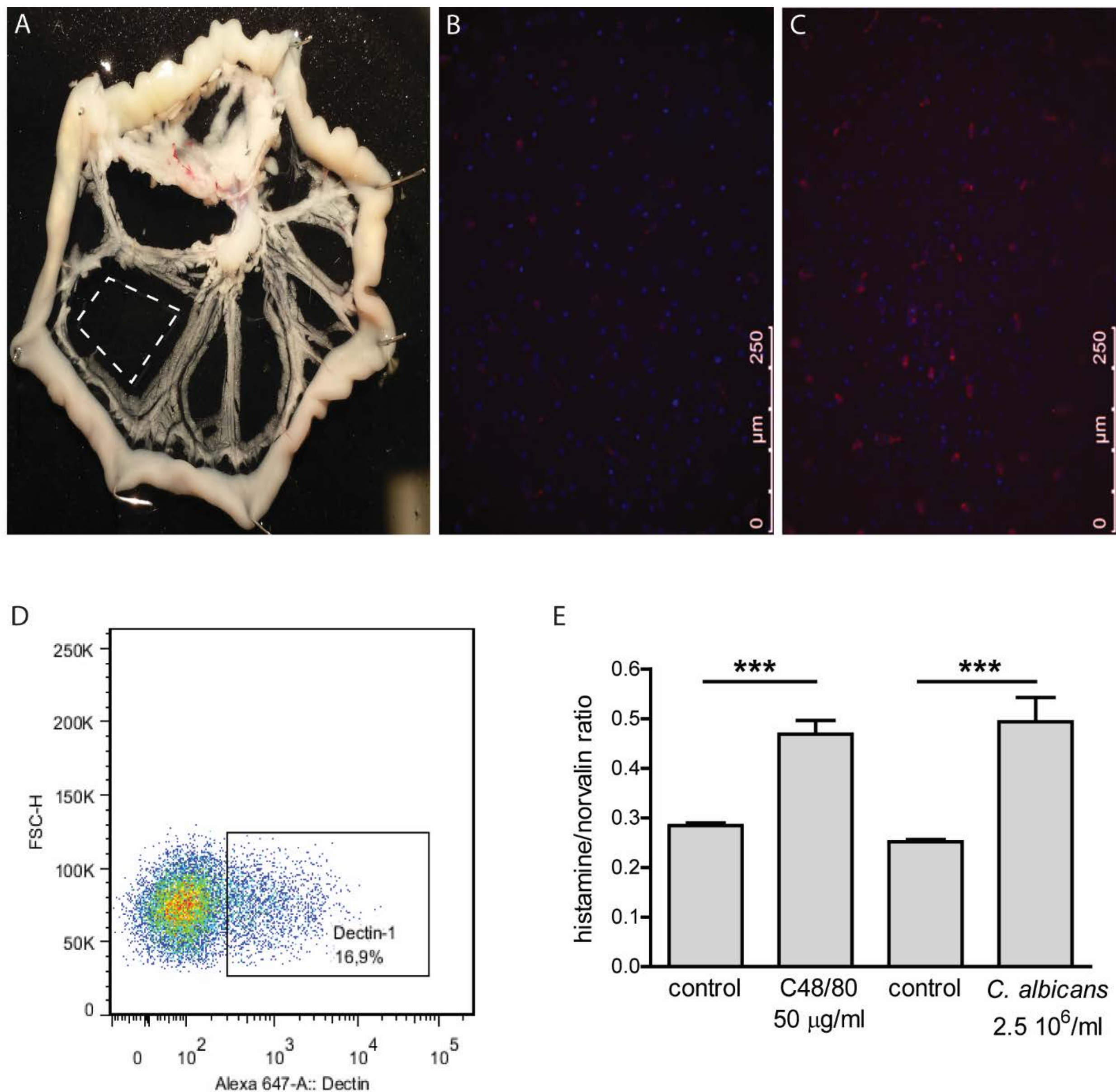
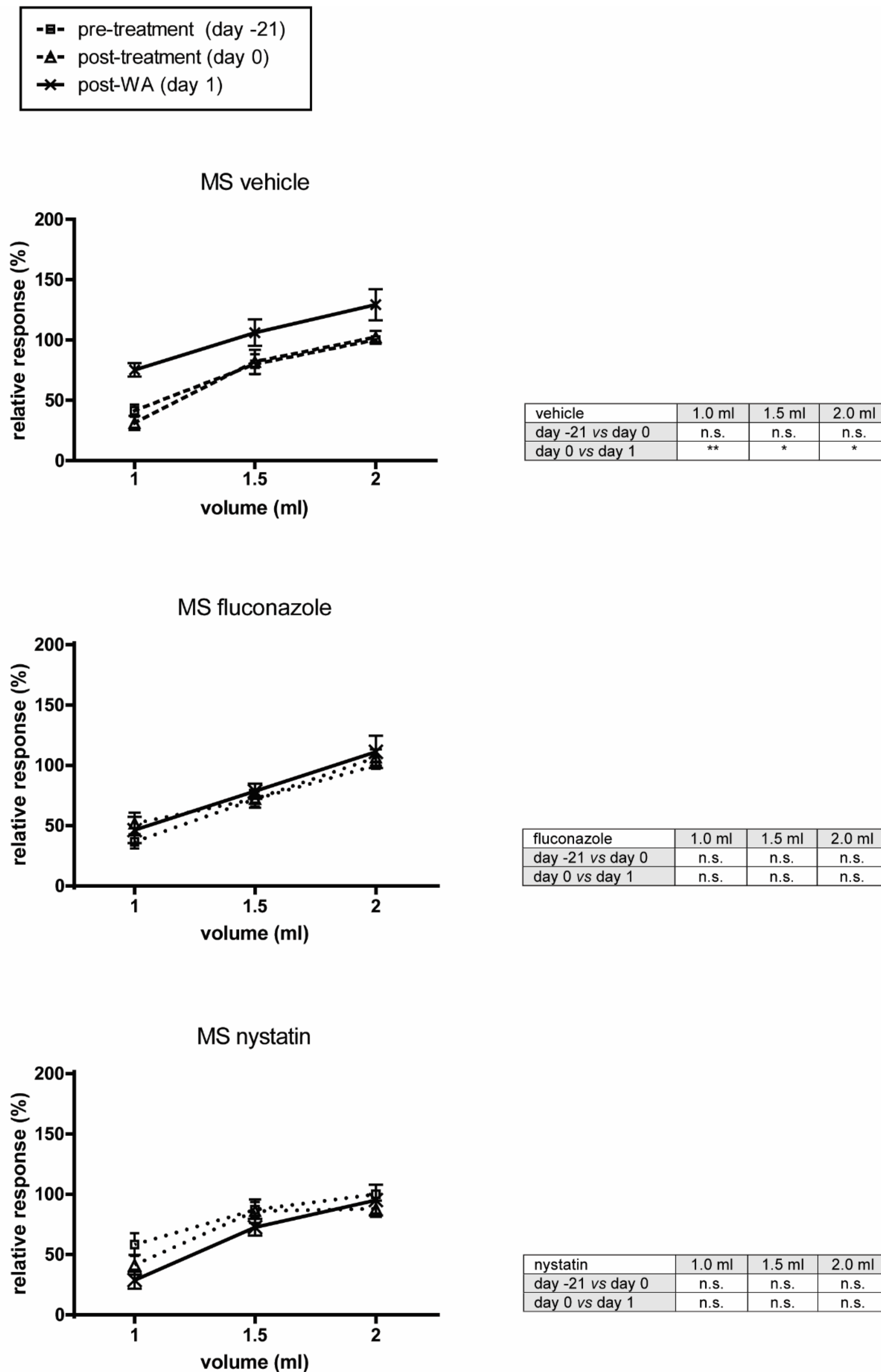


Supplementary Figure 8. Fungal induced mast cell degranulation. (A) Exteriorized mouse gut with mesentery was pinned down in Sylgard dishes. Dotted lines indicate the translucent mesenteric windows that were incubated with (B) PBS-Bovine Serum Albumin or (C) 5 mg (Dectin-1 activating) particulate β-glucan/ml PBS and stained with Texas-red labeled avidin[18] and DAPI. (D) FACS staining of the human mast cell line HMC-1 showed Dectin-1 expression in part of the cells. (E) Shows HPLC evaluation of HMC-1 cell culture supernatants.

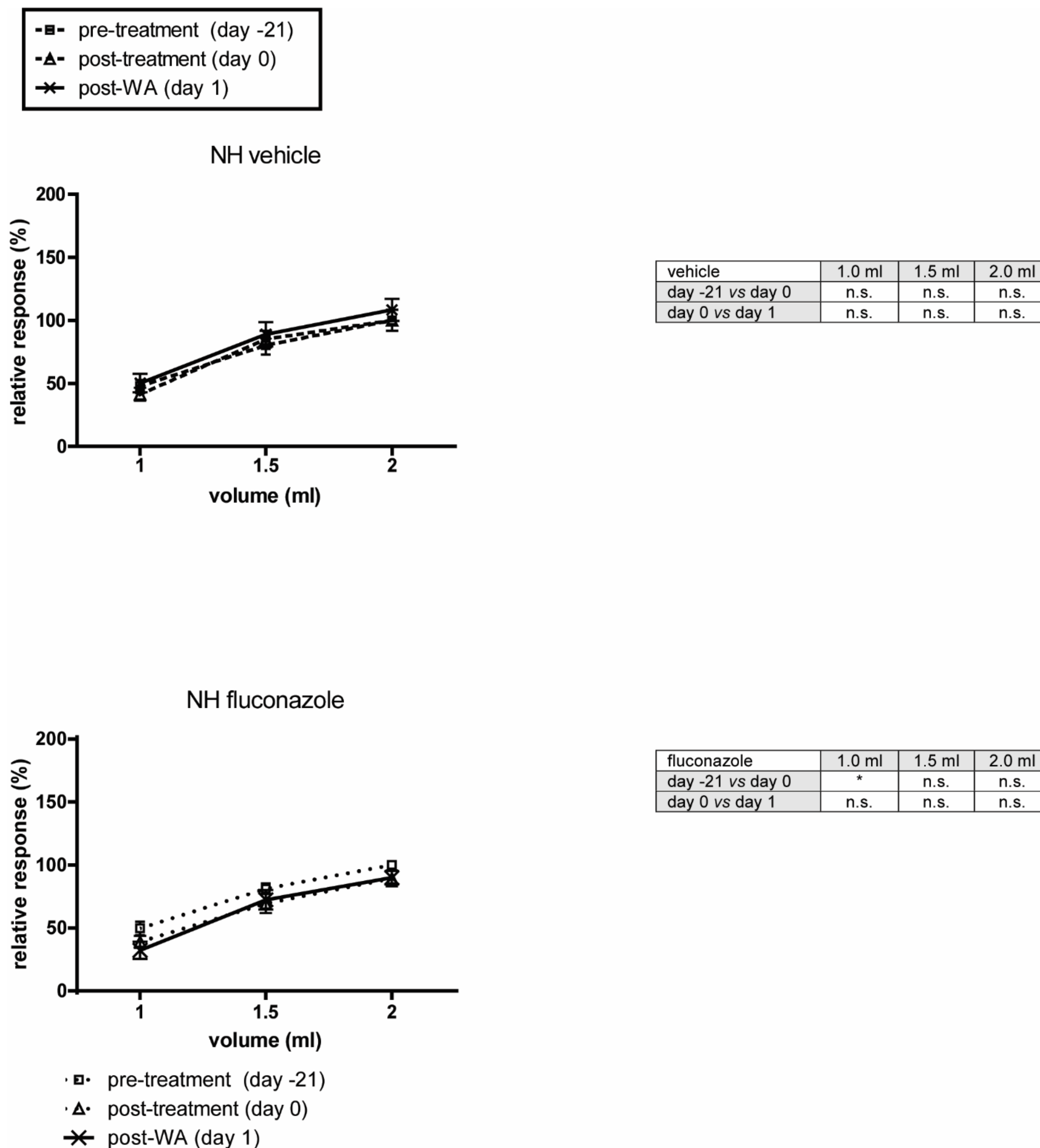


vehicle	1.0 ml	1.5 ml	2.0 ml
day -21 *vs* day 0	n.s.	n.s.	n.s.
day 0 *vs* day 1	**	*	*

fluconazole	1.0 ml	1.5 ml	2.0 ml
day -21 *vs* day 0	n.s.	n.s.	n.s.
day 0 *vs* day 1	n.s.	n.s.	n.s.

nystatin	1.0 ml	1.5 ml	2.0 ml
day -21 *vs* day 0	n.s.	n.s.	n.s.
day 0 *vs* day 1	n.s.	n.s.	n.s.

Supplementary Figure 9. Per volume line diagrams; pre water avoidance (WA) anti-fungal treatment of maternal separated (MS) rats. All data are mean ± SEM, n.s. not significant, *$P<0.05$, *$P<0.01$, n=8-9/group. Corresponding area under the curve bar diagram depicted in Figure 2A of the manuscript.



vehicle	1.0 ml	1.5 ml	2.0 ml
day -21 *vs* day 0	n.s.	n.s.	n.s.
day 0 *vs* day 1	n.s.	n.s.	n.s.

fluconazole	1.0 ml	1.5 ml	2.0 ml
day -21 *vs* day 0	*	n.s.	n.s.
day 0 *vs* day 1	n.s.	n.s.	n.s.

Supplementary Figure 10. Per volume line diagrams; pre water avoidance (WA) anti-fungal treatment of non-handled (NH) rats. All data are mean ± SEM, n.s. not significant, **P*<0.05, n=8-9/group. Corresponding area under the curve bar diagram depicted in Figure 2A of the manuscript.

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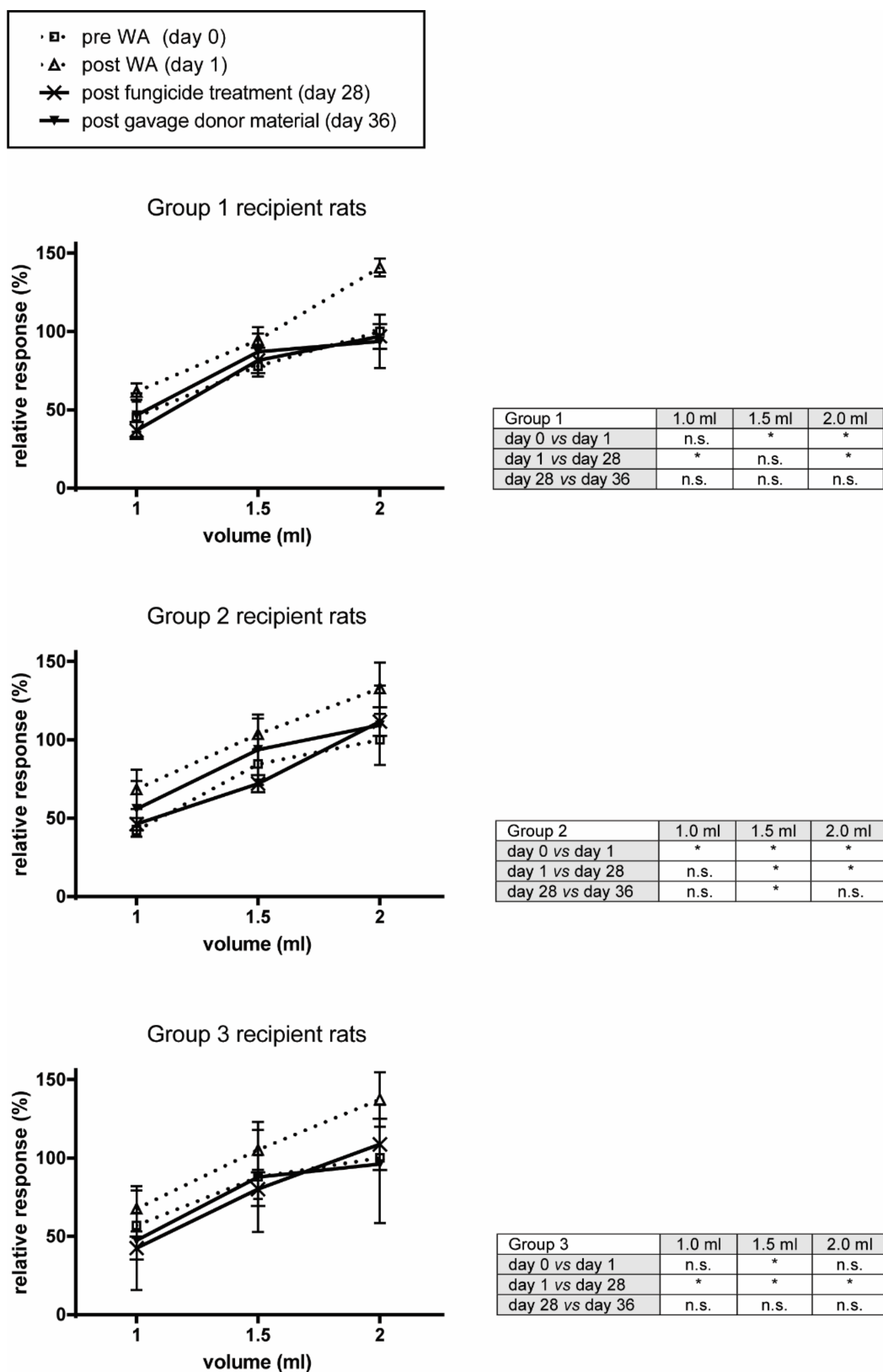

Group 1	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	n.s.	*	*
day 1 *vs* day 28	*	n.s.	*
day 28 *vs* day 36	n.s.	n.s.	n.s.

Group 2	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	*	*
day 1 *vs* day 28	n.s.	*	*
day 28 *vs* day 36	n.s.	*	n.s.

Group 3	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	n.s.	*	n.s.
day 1 *vs* day 28	*	*	*
day 28 *vs* day 36	n.s.	n.s.	n.s.

Supplementary Figure 11. Per volume line diagrams; post water avoidance (WA) anti-fungal treatment and subsequent fungal repopulation. All data are mean ± SEM, n.s. not significant, *$P<0.05$, n=8-9/group. Corresponding area under the curve bar diagram depicted in Figure 2C of the manuscript.

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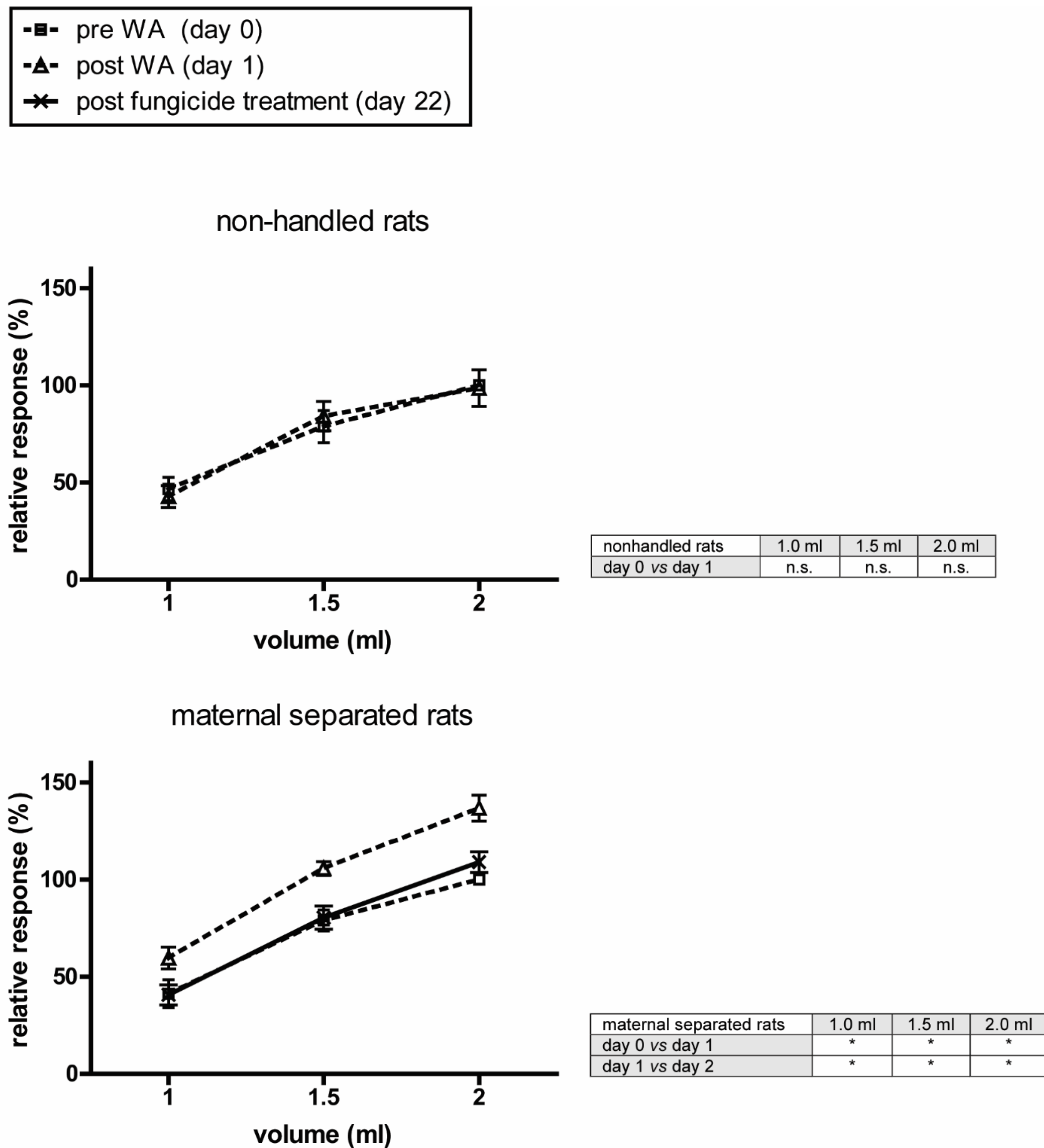
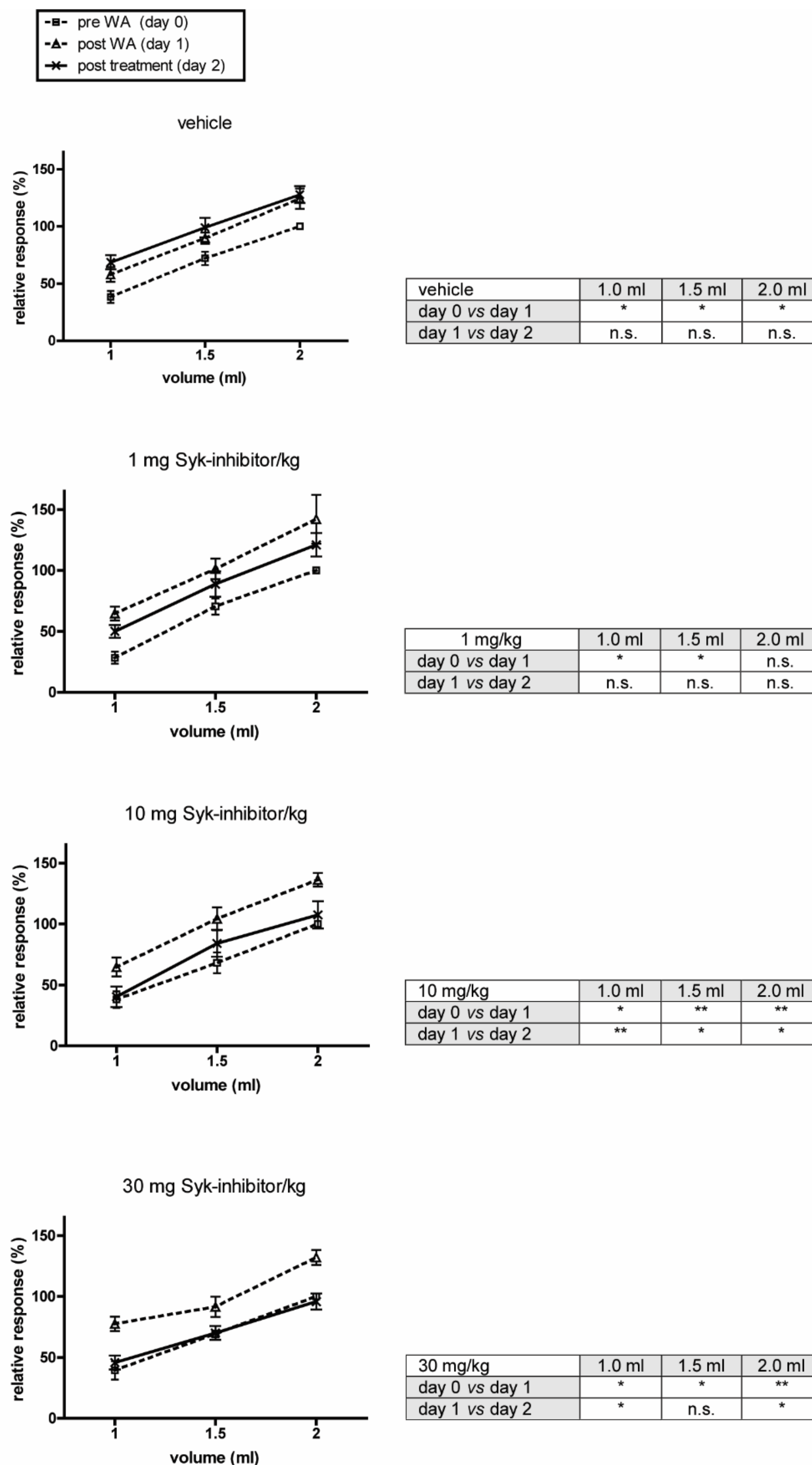


nonhandled rats	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	n.s.	n.s.	n.s.

maternal separated rats	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	*	*
day 1 *vs* day 2	*	*	*

Supplementary Figure 12. Per volume line diagrams; rats used in mycobiome evaluations. All data are mean ± SEM, n.s. not significant, **P*<0.05 (n=7-8). Corresponding area under the curve bar diagram depicted in Figure 3A of the manuscript.



vehicle	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	*	*
day 1 *vs* day 2	n.s.	n.s.	n.s.

1 mg/kg	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	*	n.s.
day 1 *vs* day 2	n.s.	n.s.	n.s.

10 mg/kg	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	**	**
day 1 *vs* day 2	**	*	*

30 mg/kg	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	*	**
day 1 *vs* day 2	*	n.s.	*

Supplementary Figure 13. Per volume line diagrams; post water avoidance (WA) treatment with Syk inhibitor. All data are mean ± SEM, n.s. not significant, **P*<0.05, ***P*<0.01, n=8-9/group. Corresponding area under the curve bar diagram depicted in Figure 6A of the manuscript.

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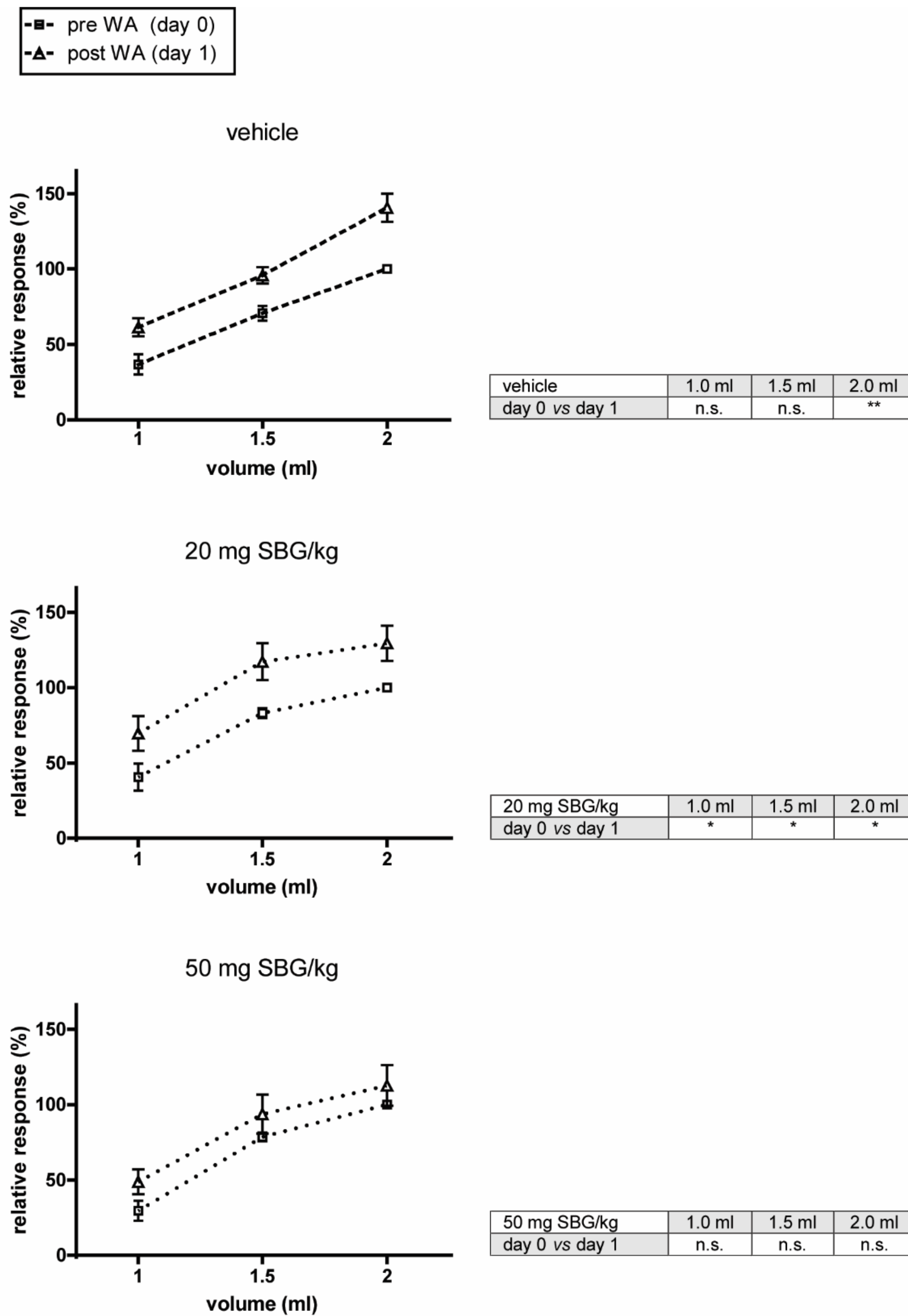
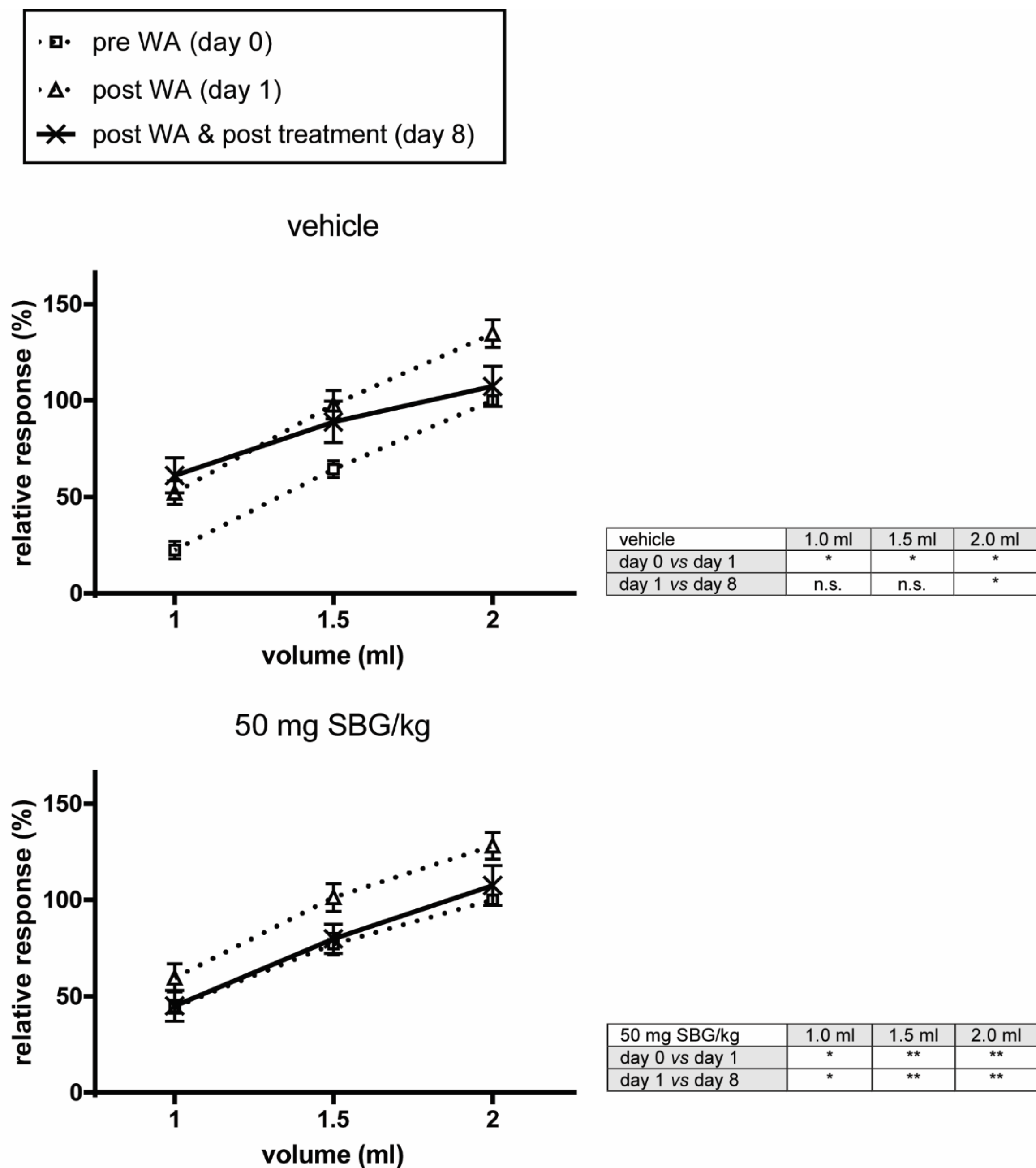


vehicle	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	n.s.	n.s.	**

20 mg SBG/kg	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	*	*

50 mg SBG/kg	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	n.s.	n.s.	n.s.

Supplementary Figure 14. Per volume line diagrams; peri water avoidance (WA) treatment with soluble β-glucans (SBG). All data are mean ± SEM, n.s. not significant, *P<0.05, **P<0.01, n=8-9/group. Corresponding area under the curve bar diagram depicted in Figure 6B of the manuscript.



vehicle	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	*	*
day 1 *vs* day 8	n.s.	n.s.	*

50 mg SBG/kg	1.0 ml	1.5 ml	2.0 ml
day 0 *vs* day 1	*	**	**
day 1 *vs* day 8	*	**	**

Supplementary Figure 15. Per volume line diagrams; post water avoidance (WA) treatment with soluble β-glucans (SBG). All data are mean ± SEM, n.s. not significant, *P<0.05, **P<0.01, n=8-9/group. Corresponding area under the curve bar diagram depicted in Figure 6C of the manuscript.

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	Hypersensitive[a] IBS n=8	**Normosensitive[a] IBS n=12**	**Healthy controls n=18**
Energy (kJ/day)	8648.9±3427.5	7997.2±2025.2	10133,4±2991.0*
Total protein (g/day)	69.6±25.3	68.9±22.8	86.9±29.7*
Total fat g/day)	79.5±38.6	76.83±28.3	93.6±28.3*
Total carbohydrates (g/day)	231.25±99.3	210.8±45.4	267,8±84.7*
Fibers (g/day)	21.13±9.4	18.8±6.7	27.7↑9.6*

Supplementary Table 1. Dietary intake based on validated food frequency questionnaire. [a)]Patient data only available for subgroups. *$P<0.05$ for Healthy controls vs IBS total (hypersensitive+normosensitive) as tested by ANOVA. No difference was found between hyper- versus normosensitive IBS patients.

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Human mycobiome	Present in rat and human	Rat mycobiome
Kazachstania telluris	Davidiella tassiana	Lasiospaeriaceae spp.
Penicillium spinulosum	Vishniacozyma victoriae	Fusarium fujikuroi complex
Penicillium commune	Alternaria metachromatica	uncultured Ascochyta
Aspergillus section Nidulantes	Epicoccum nigrum	Unclassified
Rhodosporidiobolus colostri	Trichomerium gloeosporum	Acremonium zeae
Cyberlindnera c.f. jadinii	Aspergillus section Aspergillus	Papiliotrema laurentii
Candida quercitrusa	Verticillium leptobactrum	Microdochium phragmitis
Apiotrichum gracile	Candida albicans	Fusarium poae
Acremonium spp.	Aspergillus section Nidulantes	Rhodotorula mucilaginosa
Penicillium roquefortii	Wallemia muriae	Wickerhamomyces bovis
Saccharomyces bulderi	Sporobolomyces roseus	Cryptococcus sp
Agaricus bisporus	Alternaria alternata	Acremonium sp
Candida humilis	Monographiella nivalis	Aspergillus section Aspergillus
Wickerhamomyces onychis		Claviceps purpurea
Candida parapsilosis		Wallemia sebi
Debaryomyces prosopidis		Pleospora spp.
Meyerozyma guilliermondii		Eudarluca caricis
Saccharomyces cerevisiae		Microdochium nivale var. Nivale
Phoma spp.		Cladosporium cladosporioides complex
Rhodotorula mucilaginosa		Capnodiales spp.
Penicillium section Chrysogena		Monographella albescens
Suillus luteus		Holtermanniella festucosa
Cladosporium cladosporioides complex		
Rodentomyces reticulatus		
Fusarium oxysporum complex		
Acremonium spp.		
Kazachstania barnettii		
Torulaspora delbrueckii		
Cystofilobasidium infirmominiatum		
Gloeophyllum trabeum		
Candida glaebosa		
Candida tropicalis		
Candida solani		
unclassified		
Kazachstania turicensis		

Supplementary Table 2. Mycobiome species present in rat and human fecal samples. The list of human mycobiome species was taken from the stability selection presented in figure 1C (relative weights Irritable Bowel Syndrome-hypersensitive vs healthy volunteer). The list of rat mycobiome species was taken from stability selection presented in figure S3 (relative weights maternal separated vs non-handled rats).

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