Gastroenterology 2017;153:1026–1039

BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

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

Sara Botschuijver,[1] Guus Roeselers,[2] Evgeni Levin,[2] Daisy M. Jonkers,[3] Olaf Welting,[1] Sigrid E. M. Heinsbroek,[1] Heleen H. de Weerd,[2] Teun Boekhout,[4,5] Matteo Fornai,[1,6] Ad A. Masclee,[3] Frank H. J. Schuren,[2] Wouter J. de Jonge,[1] Jurgen Seppen,[1] and René M. van den Wijngaard[1]

[1]Tytgat Institute for Liver and Intestinal Research, Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands; [2]Microbiology and Systems Biology, The Netherlands Organization for Applied Scientific Research (TNO), Zeist, The Netherlands; [3]Division Gastroenterology-Hepatology, Department of Internal Medicine, NUTRIM School for Nutrition, and Translational Research in Metabolism, Maastricht University Medical Center, Maastricht, The Netherlands; [4]Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; [5]Institute for Biodiversity and Ecosystems Dynamics (IBED), University of Amsterdam, Amsterdam, The Netherlands; and [6]Division of Pharmacology, Department of Clinical & Experimental Medicine, University of Pisa, Pisa, Italy

BACKGROUND & AIMS: Visceral hypersensitivity is one feature of irritable bowel syndrome (IBS). Bacterial dysbiosis might be involved in the activation of nociceptive sensory pathways, but there have been few studies of the role of the mycobiome (the fungal microbiome) in the 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.

Keywords: Mycobiota; Dectin-1; Immune Response; Yeast.

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 approximately 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

Abbreviations used in this paper: **AUC, area under the curve; CRF, corticotrophin releasing factor; IBS, irritable bowel syndrome; ITS, Internal Transcribed Spacer.**

Most current article

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2017.06.004

EDITOR'S NOTES

BACKGROUND AND CONTEXT

Despite vast interest for a possible role of the gut microbiome in IBS, the fungal microbiome (mycobiome) has been largely understudied.

NEW FINDINGS

Fungal dysbiosis is associated with IBS. In IBS-like rats with fungal dysbiosis, visceral hypersensitivity is reduced by the administration of fungicides or compounds interfering with fungal recognition by immune cells.

LIMITATIONS

The human mycobiome differs from the mycobiome in the rat and a causal role for fungi was addressed only in rats.

IMPACT

These findings indicate that the mycobiome may be important in IBS and should therefore be included in IBS-related gut microbiome investigations.

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] Because of gut barrier dysfunction, which 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] has largely been 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 (ie, 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 with 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.

Materials and Methods

Patient Characteristics and Ethics Statement

Patient characteristics are provided 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 with 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 1). No differences were found between overall energy intake, and intake of total protein, fat, carbohydrates, and fibers between hyper-and normosensitive patients. However, the reported intake was significantly lower in the total group of IBS patients (hyper- plus normosensitive) when compared with healthy volunteers (data not shown). Subjects included in the cohort gave written informed consent before 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°C) and humidity (45%). Non-handled and 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 no. 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 1*A* depicts the 30 most predominant species and shows that the human mycobiome is dominated

Table 1. Subject Characteristics

	Hypersensitive[a] IBS n=19	Normosensitive IBS n=20	Healthy controls n=18
Age (mean ± SD *y*)	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

*$P < .05$, **$P < .01$, ***$P < .001$ 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.
[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.
[b]Lactose-free diet.
[c]Assessed on a 5-point Likert scale.

by 2 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 2 species showed no difference when comparing the 2 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, which combines richness (number) and evenness (relative abundance) of species.[28] Figure 1*B* shows significantly higher Shannon diversity in healthy volunteers when compared with both of the IBS subgroups (hypersensitive and normally sensitive IBS; $P<.01$ and $P<.001$, respectively).

Bray Curtis dissimilarity of ITS data did not reveal well-defined clusters to separate healthy volunteer and IBS subgroups. 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 1*C* 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 1*D*). Subsequent computations of the area under the curve (AUC) showed outstanding (ie, AUC >0.9) discriminating quality. We also assessed differences between normally sensitive IBS and healthy volunteers, and

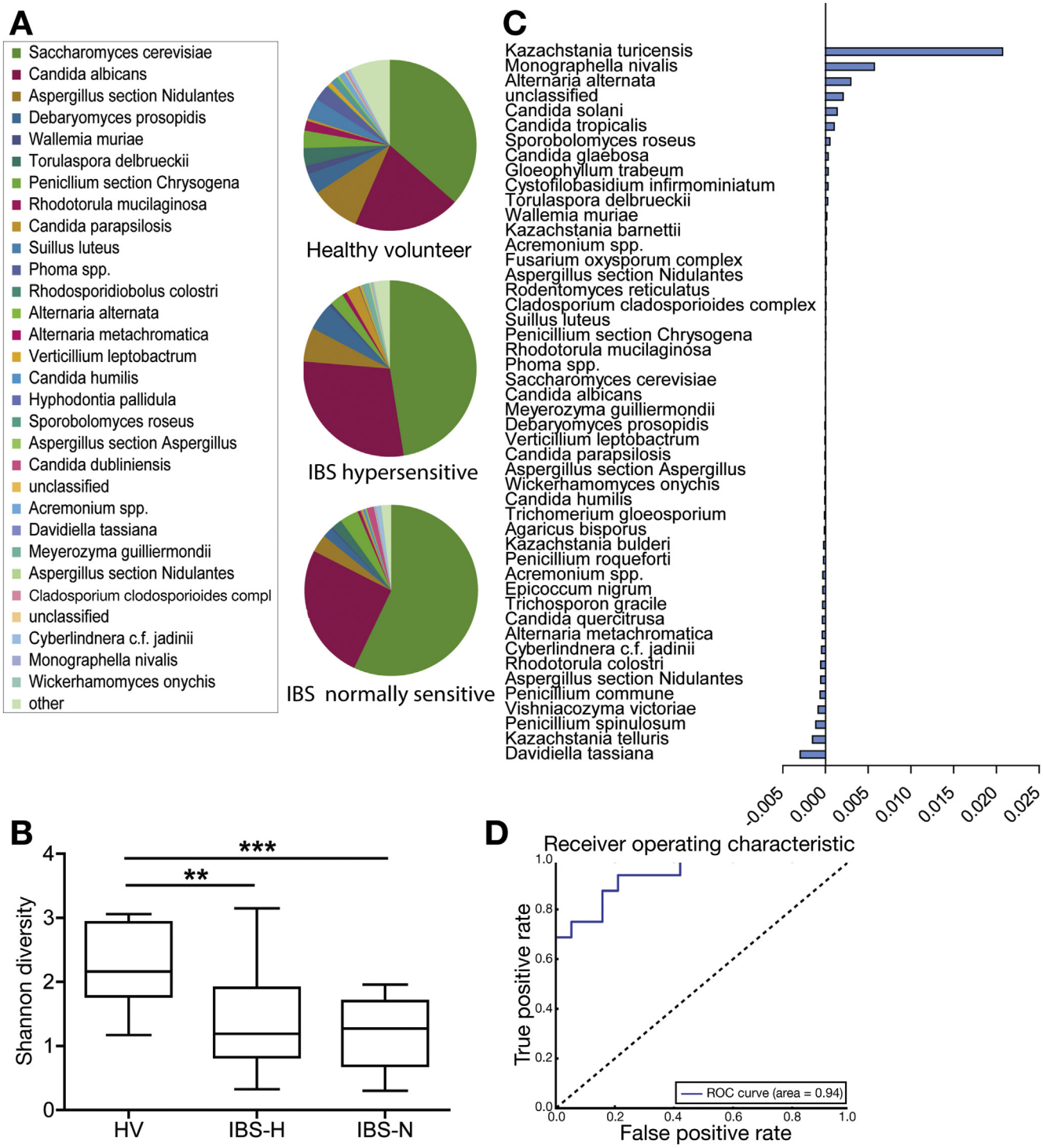


Figure 1. Differences in mycobiome between healthy volunteers (HV), hypersensitive irritable bowel syndrome (IBS-H), and normally sensitive IBS (IBS-N) patients. (*A*) Pie charts show distribution of the 30 most abundant species (+1 segment 'others') in HV, IBS-H, and IBS-N, respectively. (*B*) Shannon diversity index comparing fecal mycobiome of HV, IBS-H, and IBS-N showing median and 25%-75% and 2.5%–97.5% percentiles. N = 16–20/group, $^{**}P < .01$; $^{***}P < .001$ (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 AUC shows outstanding discriminating quality (AUC>0.9; $P < .01$).

hypersensitive IBS and normally sensitive IBS. The most robust markers of inter group differences are depicted in Supplementary Figure 3*A-B*. Corresponding receiver operating characteristic AUCs indicated good discriminating quality for these comparisons (Supplementary Figure 3*C-D*). Thus, there is altered community composition of the mycobiota in hypersensitive IBS when compared with 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 2*A*). To test the involvement of the mycobiome, 2 fungicides (fluconazole or nystatin) were administered to maternally separated rats for a 3-week period before water avoidance. Both fungicides independently prevented the occurrence of post-stress visceral hypersensitivity compared with vehicle in maternal separated rats (Figure 2*A*). 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 2*B*). 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 2*C*). All rats were then treated with a combination of fluconazole and nystatin, resulting in amelioration of visceral hypersensitivity. Next, donor

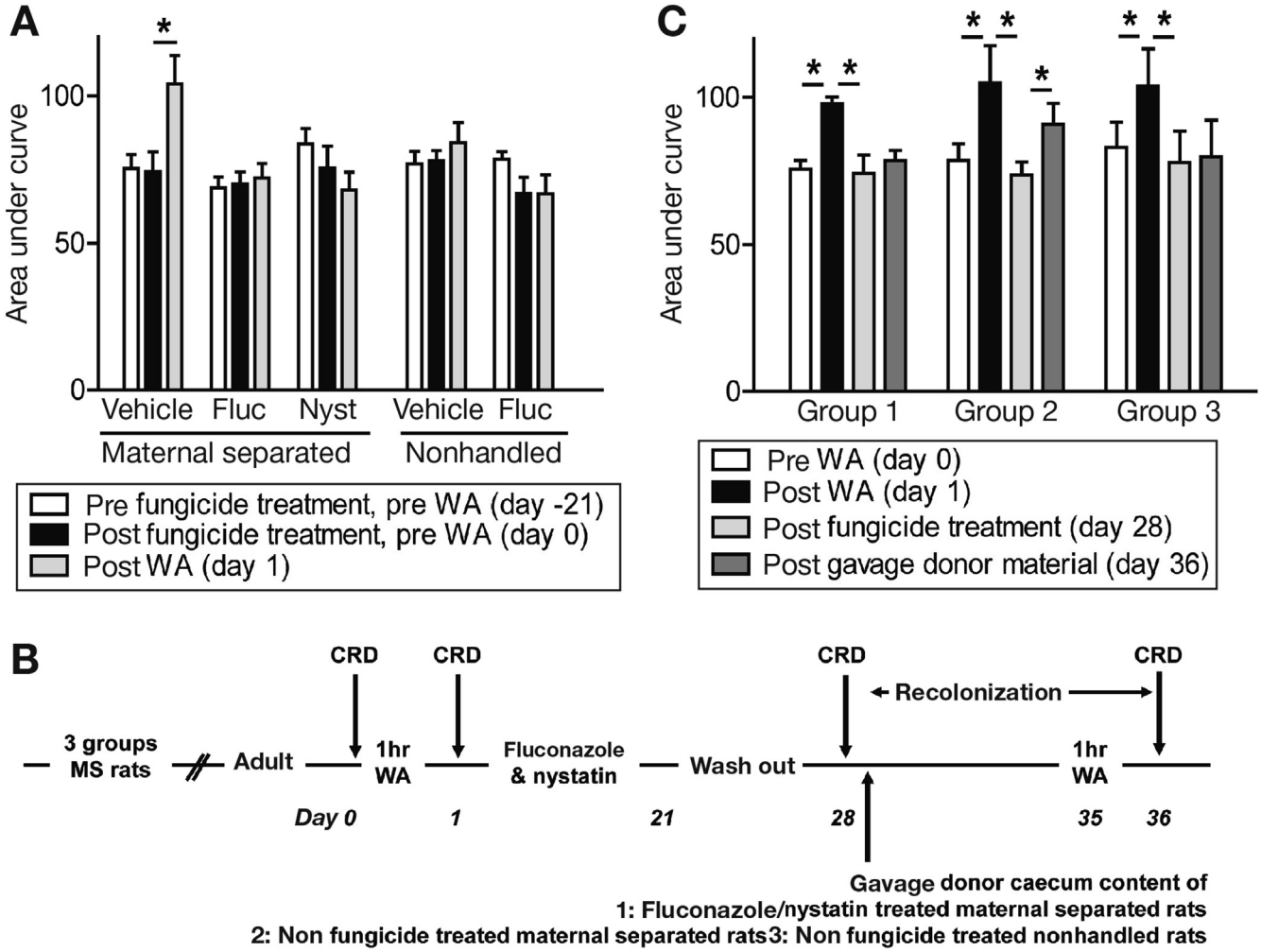


Figure 2. Fungi are important for water avoidance (WA)-induced visceral hypersensitivity of maternal separated (MS) rats. (*A*) Prevention of water avoidance-induced visceral hypersensitivity in maternal separated rats. Visceral sensitivity status is depicted by AUC of the relative response to colorectal 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 3 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, *$P < .05$ (Wilcoxon signed rank).

caecum content was administered by oral gavage. Before 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 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 Maternally Separated Rats Differs From Non-handled Rats

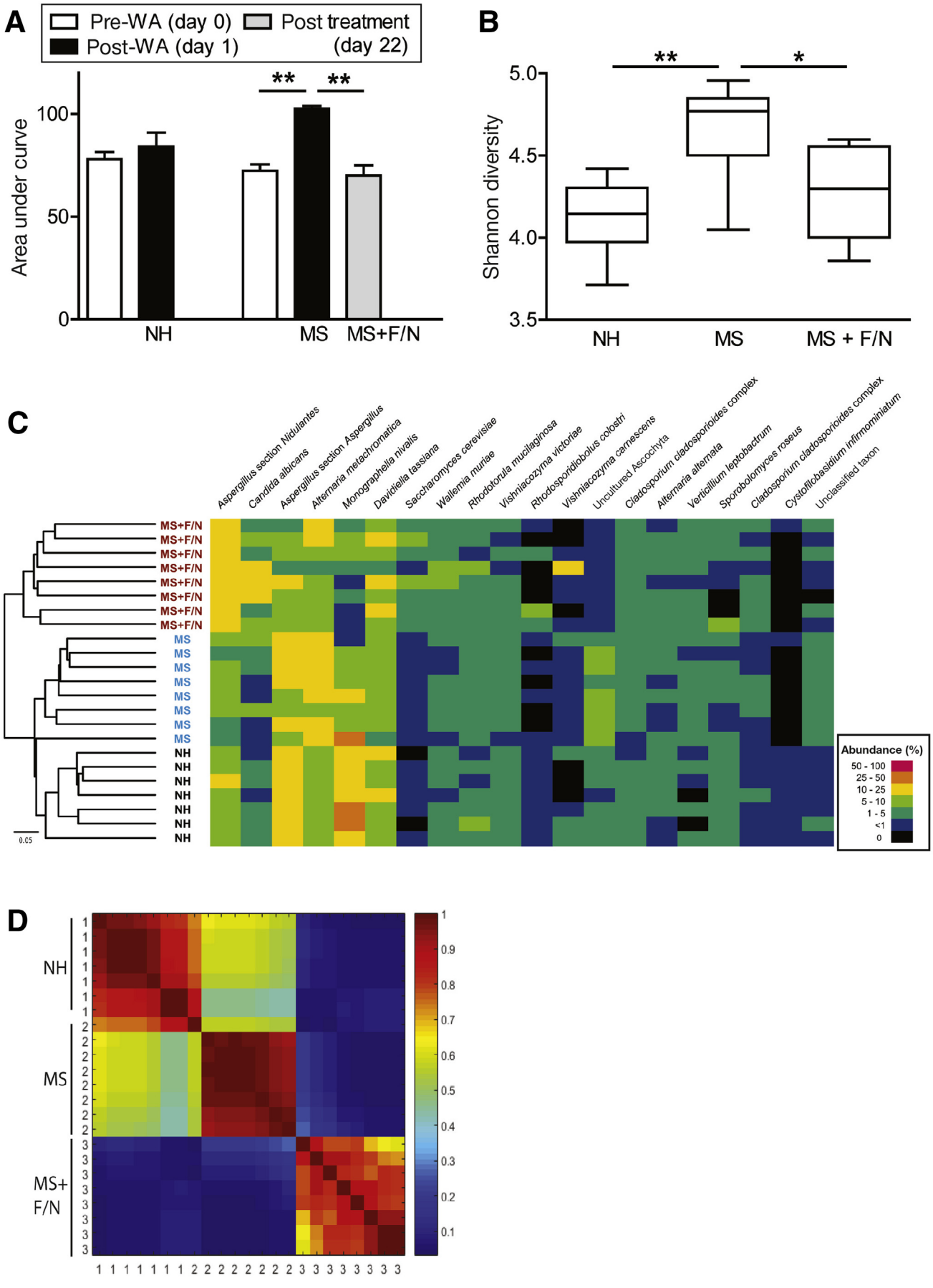
Similar to the human mycobiome, we addressed the possible differences between groups in our rat model by ITS1-based metabarcoding. We first confirmed a normal visceral sensitivity status of non-handled rats and post water avoidance hypersensitivity in maternally separated rats that was reversed upon fungicide treatment (Figure 3*A*). 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 with non-treated non-handled rats and fungicide-treated maternally separated rats (Figure 3*B*). Beta diversity (ie, 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 dendrogram of Figure 3*C* (*left*). 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 maternally 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 maternally 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 maternally 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 with 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-occurrence matrix is depicted in Figure 3*D*. 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 4*A*). Furthermore, while being on the same diet, non-handled and maternally separated rats displayed different relative species contributions (examples in Figure 4*B-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 1*C* and Supplementary Figure 6*A*, 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 5*A*). An important Syk recruiting C-type lectin receptor is Dectin-1, which 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 5*B*). 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 (twice daily gavage). Whereas vehicle-treated rats remained hypersensitive, soluble β-glucans reversed post water hypersensitivity to distension (Figure 5*C*). 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. Because the role of


A
Pre-WA (day 0)
Post-WA (day 1)
Post treatment (day 22)
Area under curve
100
50
0
**
**
NH
MS
MS+F/N
B
Shannon diversity
5.0
4.5
4.0
3.5
**
*
NH
MS
MS + F/N
C
Aspergillus section Nidulantes
Candida albicans
Aspergillus section Aspergillus
Alternaria metachromatica
Monographella nivalis
Davidiella tassiana
Saccharomyces cerevisiae
Wallemia muriae
Rhodotorula mucilaginosa
Vishniacozyma victoriae
Rhodosporidiobolus colostri
Vishniacozyma carnescens
Uncultured Ascochyta
Cladosporium cladosporioides complex
Alternaria alternata
Verticillium leptobactrum
Sporobolomyces roseus
Cladosporium cladosporioides complex
Cystofilobasidium infirmominiatum
Unclassified taxon
MS+F/N
MS
NH
0.05
Abundance (%)
50 - 100
25 - 50
10 - 25
5 - 10
1 - 5
<1
0
D
NH
MS
MS+ F/N
1 1 1 1 1 1 1 2 2 2 2 2 2 2 3 3 3 3 3 3 3
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

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 8*A-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 8*D-E*). Thus, we conclude that mast cells are indeed equipped to recognize fungi and respond with histamine release.

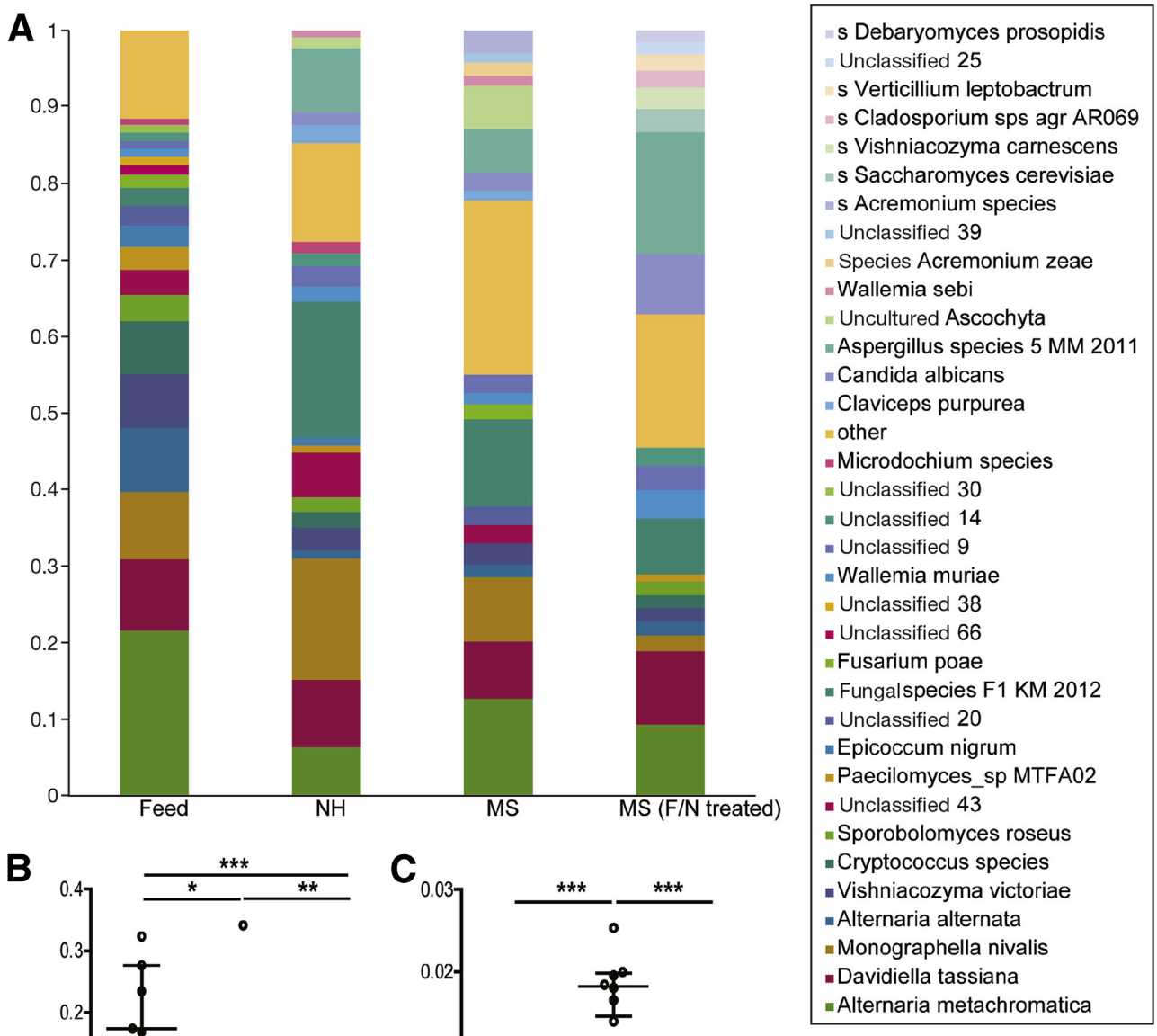
Discussion

Until now, 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 led 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.

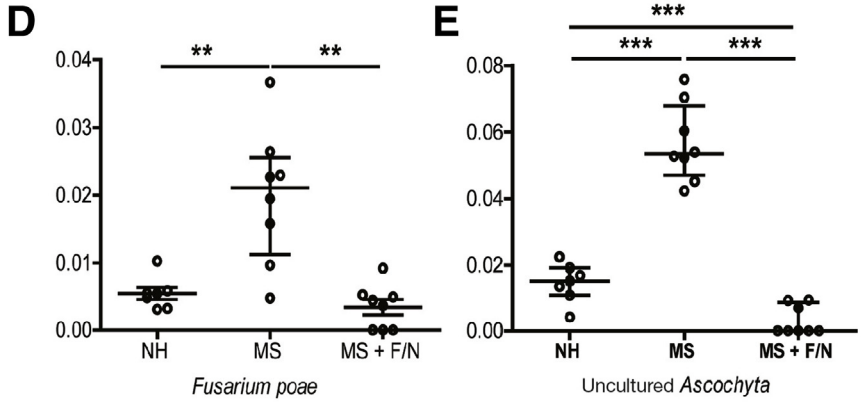
Our earlier investigations in the maternal separation model established an important role for mast cells and the histamine-1 receptor.[7,8] These results were confirmed in clinical trials,[6,9] but triggers for mast cell degranulation remained elusive. In rat, it was shown that CRF receptor antagonism 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 the mechanisms involved in the translocation of these antigens, an earlier publication by Ait-Belgnaoui et al[35] 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. 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 a similar mechanism may explain our results. Taken together, the above data suggest that post-stress visceral hypersensitivity may be the result of a 2-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 maternally separated rats, when exposed to 1 hour of water avoidance stress, remained hypersensitive to distension for at least 1 month afterwards.[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

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 AUC 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%–75% and 2.5%–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, $^{*}P < .05$; $^{**}P < .01$ (Mann Whitney and Wilcoxon signed rank).


A
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Feed
NH
MS
MS (F/N treated)
s Debaryomyces prosopidis
Unclassified 25
s Verticillium leptobactrum
s Cladosporium sps agr AR069
s Vishniacozyma carnescens
s Saccharomyces cerevisiae
s Acremonium species
Unclassified 39
Species Acremonium zeae
Wallemia sebi
Uncultured Ascochyta
Aspergillus species 5 MM 2011
Candida albicans
Claviceps purpurea
other
Microdochium species
Unclassified 30
Unclassified 14
Unclassified 9
Wallemia muriae
Unclassified 38
Unclassified 66
Fusarium poae
Fungal species F1 KM 2012
Unclassified 20
Epicoccum nigrum
Paecilomyces_sp MTFA02
Unclassified 43
Sporobolomyces roseus
Cryptococcus species
Vishniacozyma victoriae
Alternaria alternata
Monographella nivalis
Davidiella tassiana
Alternaria metachromatica

B
***
*
**
0.4
0.3
0.2
0.1
0.0
NH
MS
MS + F/N
Monographella nivalis
C
***
***
0.03
0.02
0.01
0.00
NH
MS
MS + F/N
Acremonium zeae


D
**
**
0.04
0.03
0.02
0.01
0.00
NH
MS
MS + F/N
Fusarium poae
E
***
***
***
0.08
0.06
0.04
0.02
0.00
NH
MS
MS + F/N
Uncultured Ascochyta

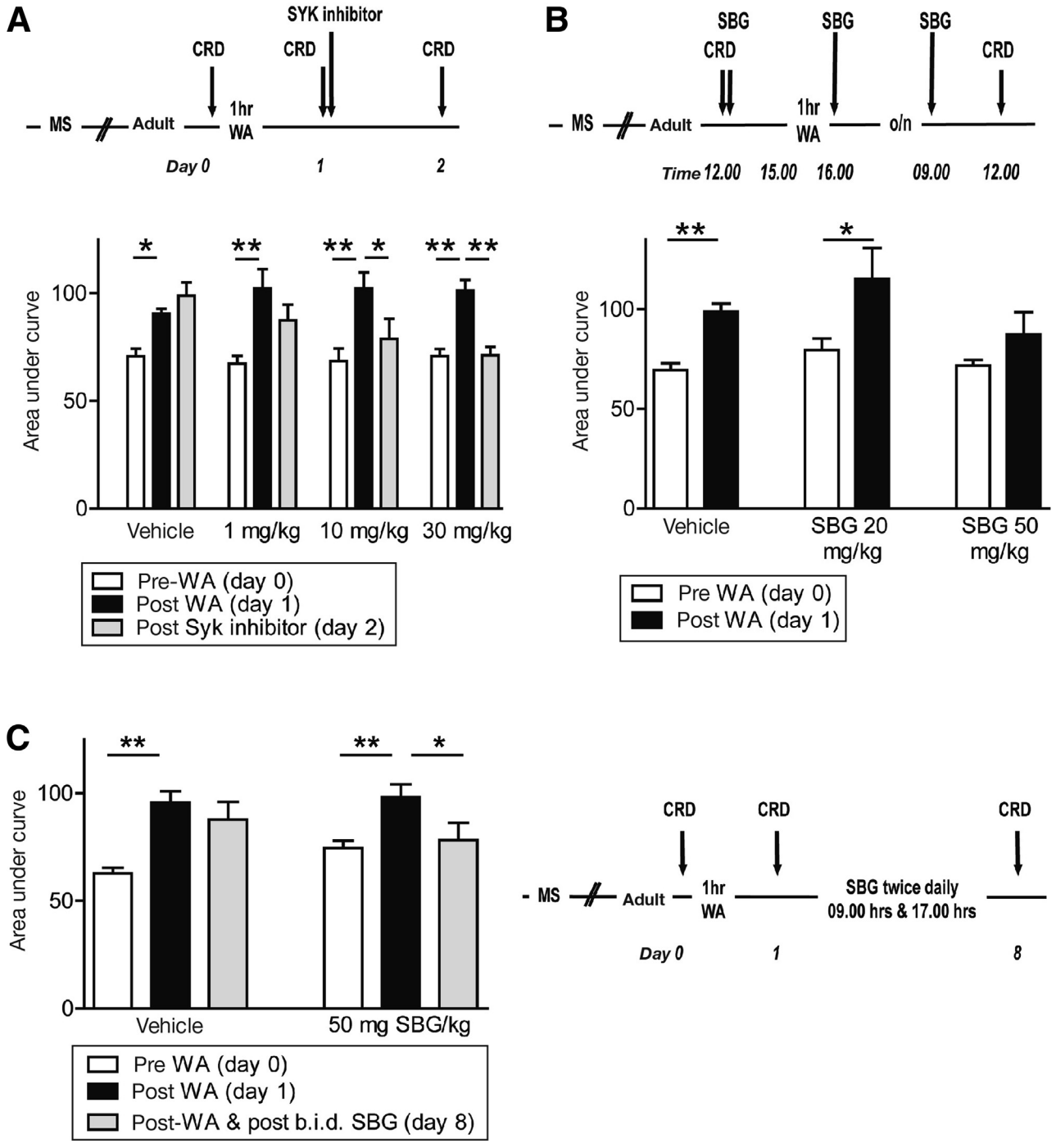


Figure 5. Fungal recognition via the Dectin-1/Syk signaling pathway is important in post water avoidance (WA)-stress visceral hypersensitivity. (*A*) Schematic representation of the experimental set-up and relative response to colorectal distension (CRD) after post water avoidance treatment with vehicle 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 (3 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 twice daily). Data are mean ± SEM. N = 8–9/group; $^{*}P < .05$; $^{**}P < .01$ (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; $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$ (Mann Whitney).

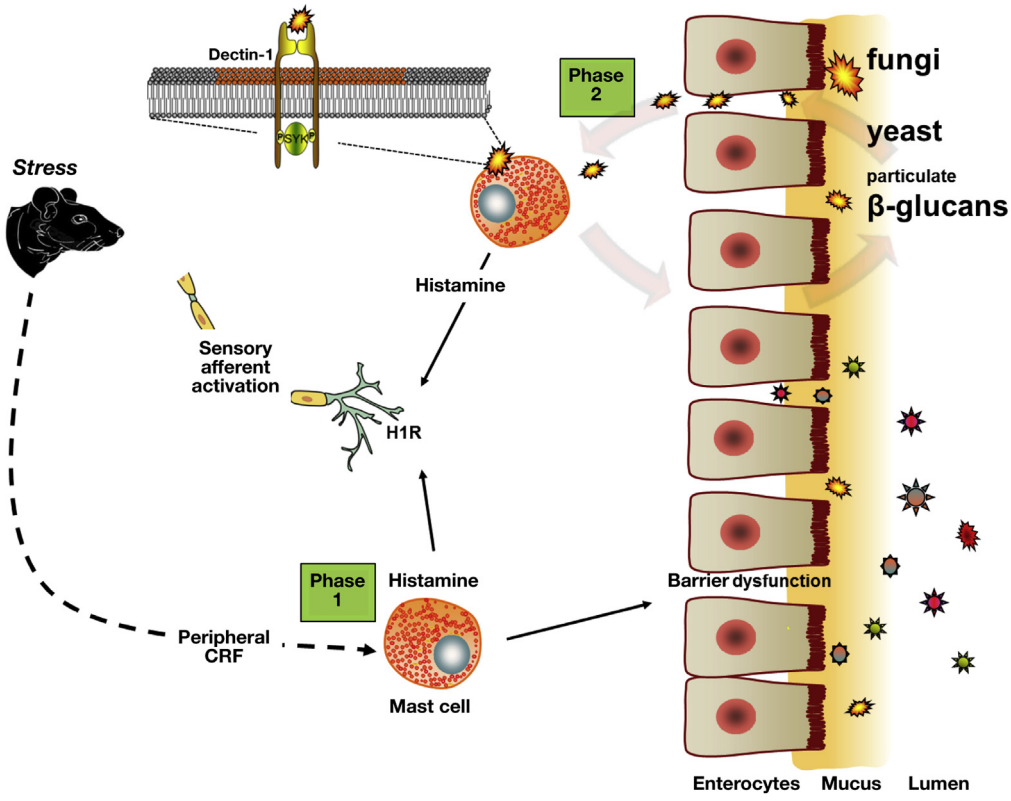


Figure 6. Proposed mechanism of the role of fungi in stress-induced visceral hypersensitivity in maternal separated rats. Previously, a key role for mast cells and the histamine-1 receptor (H1R) was evidenced in the maternal separation model. Initial stress-induced mast cell degranulation and subsequent afferent activation and barrier dysfunction (phase 1) were shown to depend on peripheral 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.

restoring visceral hypersensitivity in fungicide-treated maternally separated rats. These experiments suggested that a specific mycobiome is required and comparison of maternally separated and non-handled fecal samples indeed showed separation-induced mycobiome dysbiosis. This result confirmed our observations in humans, 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[41] showed a wide range of (1→3)-β-d-glucan levels in fungal culture supernatants. Particulate β-glucans are known to ligate Dectin-1 and possibly increased numbers of high-level β-glucan-expressing species in donor feces of maternally 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 morphologic forms and species.[39] Relevant differences may also occur on strain level. In 2007, 2 reports with seemingly opposite results were published in *Nature Immunology*. One investigation showed that Dectin-1 was essential for controlling systemic infection with *Candida albicans* in mice, the other found that Dectin-1 was not required.[42,43] In a follow-up report, 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 immunologic 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 maternally 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 with IBS subgroups. In rat, not the normal but the IBS-like phenotype instead was associated with highest α-diversity. This discrepancy possibly arises because of the relative overabundance of *Saccharomyces 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[24] found higher fungal α-diversity in fecal samples of healthy volunteers compared with inflammatory bowel disease patients. Dextran sulfate sodium (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 preclinical 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 1 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 maternally separated rats, and these fungi possibly play a role in predisposed animals only. In relation to this, De Palma et al[51] investigated the gut microbiome in relation to altered behavior. Colonization of adult germ-free maternally separated mice lead to anxiety-like behavior and behavioral despair, whereas colonization of germ-free non-handled mice did not.[51] It is possible that host factors present in maternally 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. Because of 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. However, more subtle approaches of mycobiome modulation may be suitable for IBS. This could include the use of probiotic fungal and bacterial strains that are 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.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2017.06.004.

References

1. Drossman DA. Functional gastrointestinal disorders: history, pathophysiology, clinical features and Rome IV. Gastroenterology 2016 [Epub ahead of print, http://dx.doi.org/10.1053/j.gastro.2016.02.032.
2. Elsenbruch S. Abdominal pain in irritable bowel syndrome: a review of putative psychological, neural and neuro-immune mechanisms. Brain Behav Immun 2011; 25:386–394.
3. Lovell RM, Ford AC. Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. Clin Gastroenterol Hepatol 2012;10:712–721.
4. Morgan B. Drug development: a healthy pipeline. Nature 2016;533:S116–S117.
5. Keszthelyi D, Troost FJ, Masclee AA. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. Methods to assess visceral hypersensitivity in irritable bowel syndrome. Am J Physiol Gastrointest Liver Physiol 2012;303:G141–G154.
6. Klooker TK, Braak B, Koopman KE, et al. The mast cell stabiliser ketotifen decreases visceral hypersensitivity and improves intestinal symptoms in patients with irritable bowel syndrome. Gut 2010;59:1213–1221.
7. Stanisor OI, van Diest SA, Yu Z, et al. Stress-induced visceral hypersensitivity in maternally separated rats can be reversed by peripherally restricted histamine-1-receptor antagonists. PloS One 2013;8:e66884.
8. van den Wijngaard R, Klooker T, Welting O, et al. Essential role for TRPV1 in stress-induced (mast

cell-dependent) colonic hypersensitivity in maternally separated rats. Neurogastroenterol Motil 2009;21:1107.
9. **Wouters MM, Balemans D, Van Wanrooy S**, et al. Histamine receptor H1-mediated sensitization of TRPV1 mediates visceral hypersensitivity and symptoms in patients with Irritable Bowel Syndrome. Gastroenterology 2016;150:875–887.e9.
10. Larauche M, Kiank C, Tache Y. Corticotropin releasing factor signaling in colon and ileum: regulation by stress and pathophysiological implications. J Physiol Pharmacol 2009;60(Suppl 7):33–46.
11. Van Den Wijngaard RM, Klooker TK, De Jonge WJ, et al. Peripheral relays in stress-induced activation of visceral afferents in the gut. Auton Neurosci 2010;153:99–105.
12. **Van Den Wijngaard RM, Stanisor OI**, van Diest SA, et al. Peripheral alpha-helical CRF (9-41) does not reverse stress-induced mast cell dependent visceral hypersensitivity in maternally separated rats. Neurogastroenterol Motil 2012;24:274–282; e111.
13. Dukes GE, Mayer EA, Kelleher DL, et al. A randomised double blind, placebo controlled, crossover study to evaluate the efficacy and safety of the corticotrophin releasing factor 1 (CRF1) receptor antagonist GW876008 in IBS patients. Neurogastroenterol Motil 2009;21(Suppl 1):84.
14. Sweetser S, Camilleri M, Linker Nord SJ, et al. Do corticotropin releasing factor-1 receptors influence colonic transit and bowel function in women with irritable bowel syndrome? Am J Physiol Gastrointest Liver Physiol 2009;296:G1299–G1306.
15. Piche T. Tight junctions and IBS–the link between epithelial permeability, low-grade inflammation, and symptom generation? Neurogastroenterol Motil 2014; 26:296–302.
16. Ishihara S, Tada Y, Fukuba N, et al. Pathogenesis of irritable bowel syndrome–review regarding associated infection and immune activation. Digestion 2013;87:204–211.
17. Simren M, Barbara G, Flint HJ, et al. Intestinal microbiota in functional bowel disorders: a Rome foundation report. Gut 2013;62:159–176.
18. Eisenstein M. Microbiome: bacterial broadband. Nature 2016;533:S104–S106.
19. Richard ML, Lamas B, Liguori G, et al. Gut fungal microbiota: the Yin and Yang of inflammatory bowel disease. Inflamm Bowel Dis 2015;21:656–665.
20. Santelmann H, Howard JM. Yeast metabolic products, yeast antigens and yeasts as possible triggers for irritable bowel syndrome. Eur J Gastroenterol Hepatol 2005; 17:21–26.
21. **Chehoud C, Albenberg LG**, Judge C, et al. Fungal signature in the gut microbiota of pediatric patients with Inflammatory Bowel Disease. Inflamm Bowel Dis 2015; 21:1948–1956.
22. Iliev ID, Funari VA, Taylor KD, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science 2012;336:1314–1317.
23. Qiu X, Zhang F, Yang X, et al. Changes in the composition of intestinal fungi and their role in mice with dextran sulfate sodium-induced colitis. Sci Rep 2015;5:10416.
24. Sokol H, Leducq V, Aschard H, et al. Fungal microbiota dysbiosis in IBD. Gut 2017;66:1039–1048.
25. **Lopes JP, Stylianou M**, Nilsson G, et al. Opportunistic pathogen Candida albicans elicits a temporal response in primary human mast cells. Sci Rep 2015;5:12287.
26. Nieto-Patlan A, Campillo-Navarro M, Rodriguez-Cortes O, et al. Recognition of Candida albicans by Dectin-1 induces mast cell activation. Immunobiology 2015;220:1093–1100.
27. Bjelland I, Dahl AA, Haug TT, et al. The validity of the Hospital Anxiety and Depression Scale. An updated literature review. J Psychosom Res 2002;52:69–77.
28. Jost L. Entropy and diversity. Oikos 2006;113:363–375.
29. Meinshausen N, Buhlmann P. Stability selection. J R Statistic Soc B 2010;72:417–473.
30. **Biesbroek G, Tsivtsivadze E**, Sanders EA, et al. Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. Am J Respir Crit Care Med 2014;190: 1283–1292.
31. Tsivtsivadze E, Borgdorff H, Wijgert Jd, et al. Neighborhood co-regularized multi-view spectral clustering of microbiome data. In: Zhou ZH, Schwenker F, eds. Partially supervised learning. Volume 8183. Berlin Heidelberg: Springer; 2013:80–90.
32. **Nguyen TL, Vieira-Silva S**, Liston A, et al. How informative is the mouse for human gut microbiota research? Dis Model Mech 2015;8:1–16.
33. Kerrigan AM, Brown GD. Syk-coupled C-type lectins in immunity. Trends Immunol 2011;32:151–156.
34. Brown GD, Herre J, Williams DL, et al. Dectin-1 mediates the biological effects of beta-glucans. J Exp Med 2003; 197:1119–1124.
35. Ait-Belgnaoui A, Bradesi S, Fioramonti J, et al. Acute stress-induced hypersensitivity to colonic distension depends upon increase in paracellular permeability: role of myosin light chain kinase. Pain 2005;113:141–147.
36. Barreau F, Cartier C, Ferrier L, et al. Nerve growth factor mediates alterations of colonic sensitivity and mucosal barrier induced by neonatal stress in rats. Gastroenterology 2004;127:524–534.
37. Barreau F, Cartier C, Leveque M, et al. Pathways involved in gut mucosal barrier dysfunction induced in adult rats by maternal deprivation: corticotrophin-releasing factor and nerve growth factor interplay. J Physiol 2007;580:347–356.
38. **Becker KL, Ifrim DC**, Quintin J, et al. Antifungal innate immunity: recognition and inflammatory networks. Semin Immunopathol 2015;37:107–116.
39. Underhill DM, Pearlman E. Immune interactions with pathogenic and commensal fungi: a two-way street. Immunity 2015;43:845–858.
40. **Braak B, Klooker TK**, Wouters MM, et al. Mucosal immune cell numbers and visceral sensitivity in patients with irritable bowel syndrome: is there any relationship? Am J Gastroenterol 2012;107:715–726.
41. Odabasi Z, Paetznick VL, Rodriguez JR, et al. Differences in beta-glucan levels in culture supernatants of a variety of fungi. Med Mycol 2006;44:267–272.

42. Saijo S, Fujikado N, Furuta T, et al. Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. Nat Immunol 2007;8:39–46.
43. **Taylor PR, Tsoni SV**, Willment JA, et al. Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol 2007;8:31–38.
44. **Marakalala MJ, Vautier S**, Potrykus J, et al. Differential adaptation of Candida albicans in vivo modulates immune recognition by dectin-1. PLoS Pathog 2013; 9:e1003315.
45. Park AJ, Collins J, Blennerhassett PA, et al. Altered colonic function and microbiota profile in a mouse model of chronic depression. Neurogastroenterol Motil 2013;25: 733–e575.
46. Rautava S, Luoto R, Salminen S, et al. Microbial contact during pregnancy, intestinal colonization and human disease. Nat Rev Gastroenterol Hepatol 2012;9:565–576.
47. Hallen-Adams HE, Suhr MJ. Fungi in the healthy human gastrointestinal tract. Virulence 2017;8:352–358.
48. Dollive S, Chen YY, Grunberg S, et al. Fungi of the murine gut: episodic variation and proliferation during antibiotic treatment. PLoS One 2013;8:e71806.
49. Ringel Y. The gut microbiome in irritable bowel syndrome and other functional bowel disorders. Gastroenterol Clin North Am 2017;46:91–101.
50. Crouzet L, Gaultier E, Del'homme C, et al. The hypersensitivity to colonic distension of IBS patients can be transferred to rats through their fecal microbiota. Neurogastroenterol Motil 2013;25:e272–e282.
51. De Palma G, Blennerhassett P, Lu J, et al. Microbiota and host determinants of behavioural phenotype in maternally separated mice. Nat Commun 2015; 6:7735.

Author names in bold designate shared co-first authorship.

Received September 12, 2016. Accepted June 8, 2017.

Reprint requests
Address requests for reprints to: René M. van den Wijngaard, PhD, Tytgat Institute for Liver and Intestinal Research (S2-176), Department of Gastroenterology and Hepatology, Academic Medical Center, Meibergdreef 69-71, 1105 BK Amsterdam, The Netherlands. e-mail: R.vandenWijngaard@AMC.UVA.NL.

Acknowledgements
Raw sequence data will be deposited in the European Nucleotide Archive.

Guus Roeselers, Evgeni Levin, and Daisy M. Jonkers contributed equally to is work.

The current affiliation for Guus Roeselers and Heleen H. de Weerd is: Danone-Nutricia Research, Utrecht, The Netherlands.

The current affiliations for Evgeni Levin is:Department of Experimental Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands; and Horaizon BV, Rotterdam, The Netherlands.

Conflicts of interest
The authors disclose the following: W.deJ. has received consultancy fees from GlaxoSmithKline, receives research grant support from GlaxoSmithKline, Dr Willmar Schwabe GMbH, and Mead Johnsson Nutrition Pediatric Institute. R.vdW. receives research grant support from Dr Willmar Schwabe GMbH. All other authors disclose no competing financial interests.

Funding
S.B. was supported by The Netherlands Digestive Diseases Foundation, project no. 1CDP005. W.deJ. is supported by an NWO VIDI grant and Marie Curie ETN grant no. 641665.