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Reversal of visceral hypersensitivity in rat by Menthacarin®, a proprietary combination of essential oils from peppermint and caraway, coincides with mycobiome modulation

S. Botschuijver[1] | O. Welting[1] | E. Levin[2,3] | D. Maria-Ferreira[1,4,5] | E. Koch[6] | R. C. Montijn[7] | J. Seppen[1] | T. B. M. Hakvoort[1] | F. H. J. Schuren[7] | W. J. de Jonge[1] | R. M. van den Wijngaard[1]

[1]Department of Gastroenterology and Hepatology, Academic Medical Center, Tytgat Institute for Liver and Intestinal Research, Amsterdam, The Netherlands

[2]Department of Experimental Vascular Medicine, Academic Medical Center, Amsterdam, The Netherlands

[3]Horaizon BV, Rotterdam, The Netherlands

[4]Departamento de Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, Curitiba, PR, Brazil

[5]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, PR, Brazil

[6]Dr. Willmar Schwabe Pharmaceuticals, Karlsruhe, Germany

[7]Microbiology and Systems Biology, The Netherlands Organization for Applied Scientific Research (TNO), Zeist, The Netherlands

Correspondence
R. M. van den Wijngaard, Department of Gastroenterology and Hepatology, Academic Medical Center, Tytgat Institute for Liver and Intestinal Research, Amsterdam, The Netherlands.
Email: r.vandenwijngaard@amc.uva.nl

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Abstract

Background: Irritable bowel syndrome (IBS) is a common gastrointestinal disorder associated with altered gastrointestinal microflora and increased nociception to colonic distension. This visceral hypersensitivity can be reversed in our rat maternal separation model by fungicides. Menthacarin® is a proprietary combination of essential oils from *Mentha* x *piperita* L. and *Carum carvi*. Because these oils exhibit antifungal and antibacterial properties, we investigated whether Menthacarin® can reverse existing visceral hypersensitivity in maternally separated rats.

Methods: In non-handled and maternally separated rats, we used the visceromotor responses to colorectal distension as measure for visceral sensitivity. We evaluated this response before and 24 hours after water-avoidance stress and after 7 days treatment with Menthacarin® or control. The pre- and post-treatment mycobiome and microbiome were characterized by sequencing of fungal internal transcribed spacer 1 (ITS-1) and bacterial 16s rDNA regions. In vitro antifungal and antimicrobial properties of Menthacarin® were studied with radial diffusion assay.

Key Results: Menthacarin® inhibited in vitro growth of yeast and bacteria. Water-avoidance caused visceral hypersensitivity in maternally separated rats, and this was reversed by treatment. Multivariate analyses of ITS-1 and 16S high throughput data showed that maternal separation, induced changes in the myco- and microbiome. Menthacarin® treatment of non-handled and maternally separated rats shifted the mycobiomes to more similar compositions.

Conclusions & Inferences: The development of visceral hypersensitivity in maternally separated rats and the Menthacarin®-mediated reversal of hypersensitivity is associated with changes in the mycobiome. Therefore, Menthacarin® may be a safe and effective treatment option that should be tested for IBS.

KEYWORDS
abdominal pain, bacteria, fungi, IBS, microbiome

Abbreviations: DSS, dextran sulfate sodium; IBS, irritable bowel syndrome; ITS-1, internal transcribed spacer-1; OTU, operational taxonomic unit; TNBS, trinitrobenzene sulphonic acid; TRPM8, transient receptor potential ion channel melastatin subtype 8; TRPV1, transient receptor potential cation channel subfamily v member 1; WA, water avoidance.

1 | INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic stress related functional gastrointestinal disorder characterized by abdominal pain and altered bowel habits.[1] These manifestations substantially impair IBS patient's quality of life. Despite the prevalence and impact of IBS, no evidence-based therapies covering all complaints are currently available. In particular abdominal pain is an unmet clinical need. Of the IBS patients, 35-60% show increased sensitivity of the viscera, described as a decreased threshold of discomfort to colorectal distension compared to healthy controls.[2] This hypersensitivity of the viscera is thought to represent a pathological mechanism explaining abdominal pain in this disorder. Because a clear understanding of the driving forces is currently lacking, few new drugs targeting visceral hypersensitivity are being developed. Recent investigations however, suggested a role for mycobiome dysbiosis.[3] Therefore, we hypothesized that essential oils with known fungicidal activity[4,5] may reverse fungal-induced post stress visceral hypersensitivity in rat, and that such reversal associates with changes of the gut mycobiome.

In a set of small-size clinical trials, peppermint oil was shown to significantly improve abdominal pain complaints in IBS.[6,7] Menthol, the main component, reduced symptoms of visceral hypersensitivity in mice.[5,8] Menthol was shown to act via transient receptor potential ion channel melastatin subtype 8 (TRPM8), an ion channel involved in the sensing of cold stimuli by sensory neurons.[8,9] Activated TRPM8 desensitizes transient receptor potential cation channel subfamily V member 1 (TRPV1), which is involved in IBS and visceral pain perception.[10,11] Recently, a proprietary combination of essential oils of peppermint (*Mentha* x *piperita* L.) and caraway seed (*Carum carvi*) relieved pain and discomfort in a randomized placebo controlled trial in patients with functional dyspepsia.[12] This formula, known as Menthacarin®, was also evaluated in a trinitrobenzene sulphonic acid (TNBS)-model of postinflammatory visceral hypersensitivity in rat. Adam et al[13] showed that the combined oils significantly reduced visceral hypersensitivity, whereas the individual oils were not effective. The main component of caraway oil is (+)-carvone. How (+)-carvone contributed to the analgesic effect of menthol was not addressed in this investigation but may relate to its antimicrobial activity.[14] Gut microbiome dysbiosis is a therapeutic target for visceral hypersensitivity, and bacterial dysbiosis was observed in TNBS- and dextran sulfate sodium (DSS)-induced colitis.[15-18] Recent studies in colitis models and inflammatory bowel disease (IBD) patients showed the relevance of intestinal fungi (mycobiome) for inflammation.[19-23] Whether the gut mycobiome was also relevant in the post-TNBS visceral hypersensitivity observed by Adam et al is not known, but menthol and (+)-carvone both exhibit antifungal activity.[4,5] Our own investigations in a rat model of maternal separation not only showed profound changes in gut mycobiome composition of these IBS-like rats, but also reversal of poststress visceral hypersensitivity by antifungal treatment with fluconazole and nystatin.[3] In line with these findings, we observed altered mycobiome composition in IBS patients compared to healthy controls. Consequently, we suggested that manipulation of intestinal fungi can be a treatment option for IBS-related visceral hypersensitivity.

Key Points

- The gut mycobiome was recently identified as a possible cause for abdominal pain in IBS. We investigated whether a peppermint- and caraway-oil-preparation called Menthacarin® can modulate mycobiome dependent visceral hypersensitivity in rat.
- The development of visceral hypersensitivity is associated with mycobiome changes. Menthacarin® reversed visceral hypersensitivity and concomitantly changed the mycobiome composition.
- Abdominal pain in IBS is an unmet clinical need. Our results suggest that Menthacarin® may be a natural remedy that can be evaluated in a clinical setting.

Both menthol and (+)-carvone exhibit antifungal activity, which led us to investigate whether Menthacarin®-treatment can reverse poststress visceral hypersensitivity in maternally separated rats, and if such reversal coincides with changes of the gut mycobiome. Our results suggest, that Menthacarin® alleviates abdominal pain in IBS-like rats via mycobiome modulation and we therefore think that clinical studies of Menthacarin® in IBS are warranted.

2 | METHODS

2.1 | Animals and ethics statement

Long-Evans rats (Harlan, Horst, The Netherlands) were bred at the animal facility of the Academic Medical Center (AMC, Amsterdam, The Netherlands). Non-handled and maternally separated rats were housed under open cage conditions and always bred in the same room, but never shared the same cage. 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).

2.2 | Maternal separation

Neonatal maternal separation in Long Evans rats predisposes for stress-induced visceral hypersensitivity at adult age.[24] This model mimics early life events shown to be associated with risks of IBS later in life.[25] From postnatal day 2 to 14, dams were separated from the nest for 3 hours daily. During separation, dams were placed in another room and the cage with litter was placed on a heating mat to maintain pup body temperature. Non-handled pups were nursed normally. Pups were weaned at day 22 and only male rats were used in our investigations.

2.3 | Colonic distension protocol and water avoidance

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.[3,10,24] In short, the balloon catheter was inserted under short isoflurane anesthesia and, after 20 minute recovery, colonic distension was achieved by inflation of graded volumes of water (1.0, 1.5, and 2.0 mL). Length and diameter of the balloon during a 2 mL maximum volume distension were 18 and 15 mm respectively. After each 20 second distension period, water was quickly removed and an 80 second resting period was observed. For 1 hour water avoidance (WA) stress at adult age, rats were positioned on a pedestal attached to the bottom of a plexiglass tank that was filled with water within 1 cm of the top of the pedestal.

2.4 | Measurement of the visceromotor response to colonic distension and data analysis

Colorectal distension in rat leads to reproducible contractions of abdominal musculature; the so called visceromotor response. We used quantification of these contractions by radio telemetric electromyography to assess visceral pain. Further details on this technique have been published extensively.[3,10,24] Data were acquired with AcqKnowledge software (Biopac Systems Inc., Goleta, CA, USA) and then analyzed. Each 20 second distension period and its preceding 20 second of baseline recording were extracted from the original raw data, corrected for movement and breathing, rectified and integrated. Absolute datasets were then obtained by subtracting the 20 second baseline recording from the 20 second distension result. 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.

2.5 | Treatment protocol

Rats were subjected to three colorectal distension protocols during which the visceromotor response was measured; directly before WA (day 0), 1 day after WA (day 1), and 7 days after WA (day 8). Menthacarin® (Dr. Willmar Schwabe GmbH, Karlsruhe, Germany) or control was administered by oral gavage between day 1 and 8 at approximately 11:00 hours daily. Menthacarin® contains a proprietary combination of essentials oils of specified quality from *Mentha* x *piperita* L. (WS® 1340) and *Carum carvi* (WS® 1520) in a ratio of 1.8:1. Dosages were chosen on the basis of allometric dose translation according to Reagon-Shaw et al[26] In accordance with the recommended single dose in human[12], an equivalent dose in rat equals about 12.5 mg kg^{-1}. Based on this, dosages which correspond to once, twice or three times the average human equivalent single dose were administered to rat. Accordingly, four groups of maternally separated rats received 12.5, 25, or 75 mg Menthacarin® per kg in the mixture of control tryglycerides, or control (2 mL kg^{-1}). A mixture of triglycerides of saturated fatty acids (mainly caprylic and capric acid) extracted from palm oil according to European Pharmacopoeia was used as control substance as these compounds have been observed to be most suitable for the solubilization of the lipophilic essential oils. Besides, this mixture is well-tolerated after oral application and saturated fatty acids are also used as excipient in the therapeutically used peppermint- and caraway-oil-preparation. Two groups of non-handled rats received 75 mg Menthacarin® per kg or control. Each experimental group consisted of nine animals. Fecal samples were collected directly from the anus just prior to distension protocols at day 1 and day 8, snap frozen and stored at −80°C.

2.6 | DNA extraction

DNA for myco- and microbiome analysis was isolated from fecal samples collected at day 1 post-WA and day 8 post-WA. We evaluated three groups: control treated maternally separated rats, high dose (75 mg kg^{-1}) Menthacarin® treated maternally separated rats and high dose Menthacarin® treated non-handled rats. The DNA isolation procedure was carried out as described earlier.[3]

2.7 | Sequencing of fungal ITS amplicons

Barcoded fungal internal transcribed spacer regions (ITS) amplicons were generated using a 2-step PCR approach identical to our previous investigations.[3] In short, fungal ITS 1 regions were first amplified with the following primers: forward 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and reverse 5′-GCTGCGTTCTTCATCGATGC-3′. 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).[27] Reactions were cleaned and dual barcodes (8 bp) and Illumina sequencing adapters were attached using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) according to manufacturer's protocols. Barcoded amplicons were quantified, normalized to the same concentrations, pooled, and gel purified. 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.[28] Unique sequences were taxonomically classified by the RDP-II Naïve Bayesian Classifier[29] using a 60% confidence threshold against the Mothur formated UNITE Database (version no. 7).[30]

The raw sequence data generated in this study will be deposited in the European Nucleotide Archive upon acceptance of the manuscript.

2.8 | Bacterial 16S sequencing

Analysis of rat microbiome composition was performed by 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 (1 ng) to prevent over-amplification. These amplicons, generated using adapted primers 533F and 806R, were bidirectionally sequenced using the MiSeq system (Illumina) as described previously.[31] All data extraction, preprocessing, analysis of operational taxonomic units (OTUs) and classifications were performed using modules implemented in the Mothur software platform as in Kelder et al[32] except where noted below. A total of 3 741 564 high-quality sequences were aligned using the "align.seqs" command and the Mothur-compatible Bacterial SILVA SEED database. A total of 8925 unique sequences were retrieved using this pipeline. Sequences were clustered in 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. Sequences were normalized to 17 500 sequences per sample (subsampling method).

2.9 | Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 16.0; SPSS Inc., Chicago, IL, USA) or GraphPad Prism (version 7.02; Graphpad software, San Diego, CA, USA). Visceromotor response data were analyzed by 2-way ANOVA with Tukey's correction for multiple comparisons. Antimicrobial activity was analyzed with one way ANOVA repeated measurements and Dunnet's post hoc test. Relative abundances of fungal species or bacterial phyla or genera were compared using the Kruskal–Wallis statistic and Dunn's post hoc test. *P* values <.05 were considered statistically significant in all tests.

2.10 | Microbiome and mycobiome visualization

Classical clustering based on Bray–Curtis similarity and the UPGMA algorithm, non-metric multidimensional scaling based on Bray–Curtis similarity, and the Shannon diversity index were performed in the free software platform Past v3.034. Indices concerning the mycobiome were designed from taxonomically classified unique sequences at the species level. Indices concerning the microbiome were designed on OTU abundance. Resulting Shannon indices were compared with Kruskal–Wallis statistic and Dunn's post hoc test.

Similar to our previous investigations, an unsupervised co-regularized spectral clustering algorithm was applied to the ITS (species level) and 16S (genus level) datasets.[3,33,34] In short, this multi-view clustering algorithm allows for identification of clusters of 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 that have been reported to notably outperform standard techniques (e.g. k-means, hierarchical clustering, etc.) in clustering accuracy and stability.[35] 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.

2.11 | Antifungal and antibacterial properties of Menthacarin®

Agar well diffusion assays were used to confirm antifungal and antibacterial properties of peppermint oil, caraway oil, and Menthacarin®.[4,5,14,36] All three samples were tested in triplicate in concentrations of 5, 10, and 20 mg mL^{-1}. For the antifungal assay an aliquot of an overnight culture of *Candida albicans* was cultured in Sabouraud broth for 3 hours, diluted in top agar (10^5 counts per mL in 0.6% agarose in Sabouraud), and spread in 20 mL aliquots in petri dishes. After solidification, 3 mm holes were punched and filled with 8 µL sample or control that were all diluted in 40% ethanol to obtain appropriate concentrations. Fluconazole (250 µg mL^{-1}) was used as positive control. Plates were incubated 24-48 hours and the diameter of the inhibition halo was measured with a caliper. For the antibacterial assay, an aliquot of an overnight culture of *Bacillus subtilis* was cultured in 3% tryptic soy broth to an OD of 0.800 and then diluted 10 times in 0.3% TBS/1% Agar/0.02% Tween. 15 mL aliquots of this suspension were spread in Petri dishes. After solidification, 3 mm holes were punched and filled with 8 µL sample, control or a broad spectrum antibiotic mixture containing penicillin and streptomycin (1000 IU mL^{-1}) that was used as positive control. After diffusion into the plates (3 hours at 37°C), dishes were covered with a top layer (6% TBS/1% agar) and further incubated for 18-24 hours where upon the diameter of the inhibition halo was measured. Importantly, peppermint oil, caraway oil, and Menthacarin® are probably not effective against all fungal and bacterial species. *C. albicans* and *B. subtilis* merely served as proof of principle targets for fungicidal and bactericidal potential, their choice does not necessarily reflect importance for visceral pain.

3 | RESULTS

3.1 | Menthacarin® reverses stress-induced visceral hypersensitivity in maternally separated rats

Menthacarin®'s effect on visceral sensitivity was studied in maternally separated and non-handled rats (setup depicted in Figure 1A). Results are shown as area under the curve of the relative responses. Compared to pre-WA data, all maternal separation groups showed significantly increased post-WA response to distension (Figure 1B, day 0 vs day 1), except for the 12.5 mg kg^{-1} treatment group. The latter may be caused by the relatively high baseline sensitivity to distension (day 0) of this particular group. The enhanced day 1 response was reversed after 1 week treatment with 25 and 75 mg Menthacarin® per kg, but not by 12.5 mg kg^{-1} and control (Figure 1B, day 1 vs day 8). In non-handled rats, only control and high dose Menthacarin® (75 mg kg^{-1}) were evaluated. Average area under the curve data (Figure 1C) showed no changes in pre- vs 24 hours post-WA sensitivity to distension. Similarly, no changes were observed after 7 days Menthacarin® or control treatment. Thus, WA only induced visceral hypersensitivity in maternal separated rats, and this response was reversed by Menthacarin® treatment.

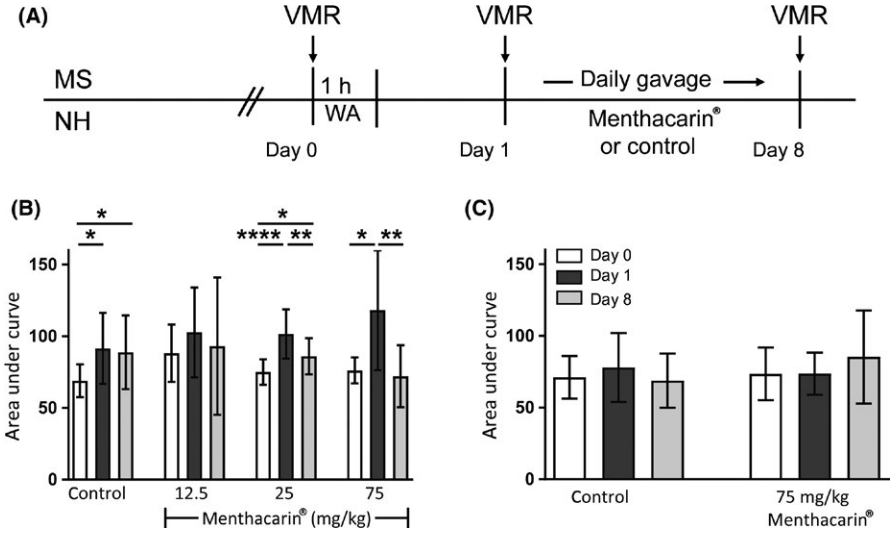

FIGURE 1 Menthacarin® mediated reversal of postwater avoidance (WA) visceral hypersensitivity in maternally separated rats. A, Experimental set up for 7 day post WA Menthacarin® or control treatment in maternal separation (MS) or non-handled (NH) rats. The visceromotor response (VMR) to colorectal distension was measured at day 0, day 1 and day 8. B, Visceral sensitivity status of maternally separated rats is depicted by area under the curve of the relative response to distension. C, Relative response to distension in non-handled rats. All data are mean ± SD, **P* < .05, ***P* < .01, *****P* < .0001, N = 9/group

3.2 | Menthacarin®, peppermint oil and caraway oil have in vitro antimicrobial properties

Peppermint oil, caraway oil, and Menthacarin® were evaluated in in vitro radial diffusion assays, using the yeast *C. albicans* and bacterial *B. subtilis* as target species. Their inhibitory potential was compared to the negative control and the assay was validated with the fungicide fluconazole or a broad spectrum antibiotic mixture containing penicillin and streptomycin. In *C. albicans* seeded agar (Figure 2A), the inhibitory activity of fluconazole justified the use of the radial diffusion assay as antifungal readout. When compared to control, peppermint oil and Menthacarin® both induced inhibition of growth at 20 mg mL^{-1}, whereas caraway oil induced growth inhibition at 5, 10, and 20 mg mL^{-1}. In *B. subtilis* seeded agar (Figure 2B), the combination of penicillin and streptomycin effectively inhibited growth when compared to control. Peppermint oil inhibited growth at 10 and 20 mg mL^{-1} and caraway oil and Menthacarin® at all concentrations tested. Taken together, these results show profound antimicrobial and antifungal activities of peppermint oil, caraway oil, and Menthacarin®. Figure 2C shows typical examples of radial diffusion assays.

3.3 | Menthacarin® treatment affects maternal separation induced mycobiome dysbiosis

To address possible changes of the fecal mycobiome, we used high throughput sequencing of fungal ITS-1 18S rDNA regions. As an aid to distinguish normosensitive (blue) and hypersensitive (red) animals depicted in Figures 4-7, Figure 3 shows a schematic representation of color coded sample groups 1-6. From a total of 54 fecal samples, six isolates divided over five groups did not contain enough DNA for subsequent analysis. Our sequencing approach generated 4 976 036 unique paired reads, which were subsequently classified into 109 fungal species whilst 30 remained unclassified.

Fungal α diversity between groups was calculated by Shannon diversity index at species level. At baseline (i.e. day 1 after WA), we observed increased α diversity in hypersensitive maternally separated rats compared to normally-sensitive non-handled rats (Figure 4A, group 1 vs group 3). This result confirmed our recently published findings.[3] No significant changes in α diversity were observed upon treatment with Menthacarin® or control. In addition, when using normalized data to compare the relative presence of the five most abundant fungal classes (Figure 4B), there were no differences between treatment groups. To investigate whether intestinal fungal composition correlated with sensitivity status and treatment, we performed a co-occurrence spectral analyses. In this unsupervised machine learning based approach, rats are clustered based on similarity of the multivariate ITS-1 composition. A symmetric heat map of the resulting sorted co-occurrence matrix is given in Figure 4C. Based on high co-occurrence values, five separate clusters can be recognized. Spreading over clusters was observed for most of the different treatment groups. Nevertheless, the pretreatment mycobiome of 6 of 9 non-handled (group 1) rats clustered in the second left quadrant. This set them apart from the pretreatment maternal separation rats (i.e. groups 3, 5, and 6) that mostly clustered in the lower three quadrants. The simultaneous presence of pre- and postcontrol treated maternal separation rats (groups 5 and 6) in the lower right quadrant demonstrated that control treatment did not affect the gut mycobiome. Most importantly however, the upper left quadrant showed clustering of 13 of 15 non-handled and maternally separated rats subjected to Menthacarin® treatment (groups 2 and 4). Thus, non-handled and maternally separated rats showed dissimilar mycobiota prior to treatment, but Menthacarin® shifted both groups to more similar mycobiomes.

We then sought to corroborate these findings with a β-diversity index, and performed classical clustering using the Bray–Curtis dissimilarity index and the UPGMA algorithm (Figure 5A). At 0.48 similarity, the dendrogram showed three separate branches, excluding only one rat. The right-hand branch almost exclusively consisted of normally sensitive rats, i.e. non-handled and maternally separated rats that had been subjected to Menthacarin® treatment (groups 2 and 4). The middle branch encompassed most of the hypersensitive rats, i.e. maternally separated rats that were sampled prior to Menthacarin® or control treatment and maternally separated rats sampled after

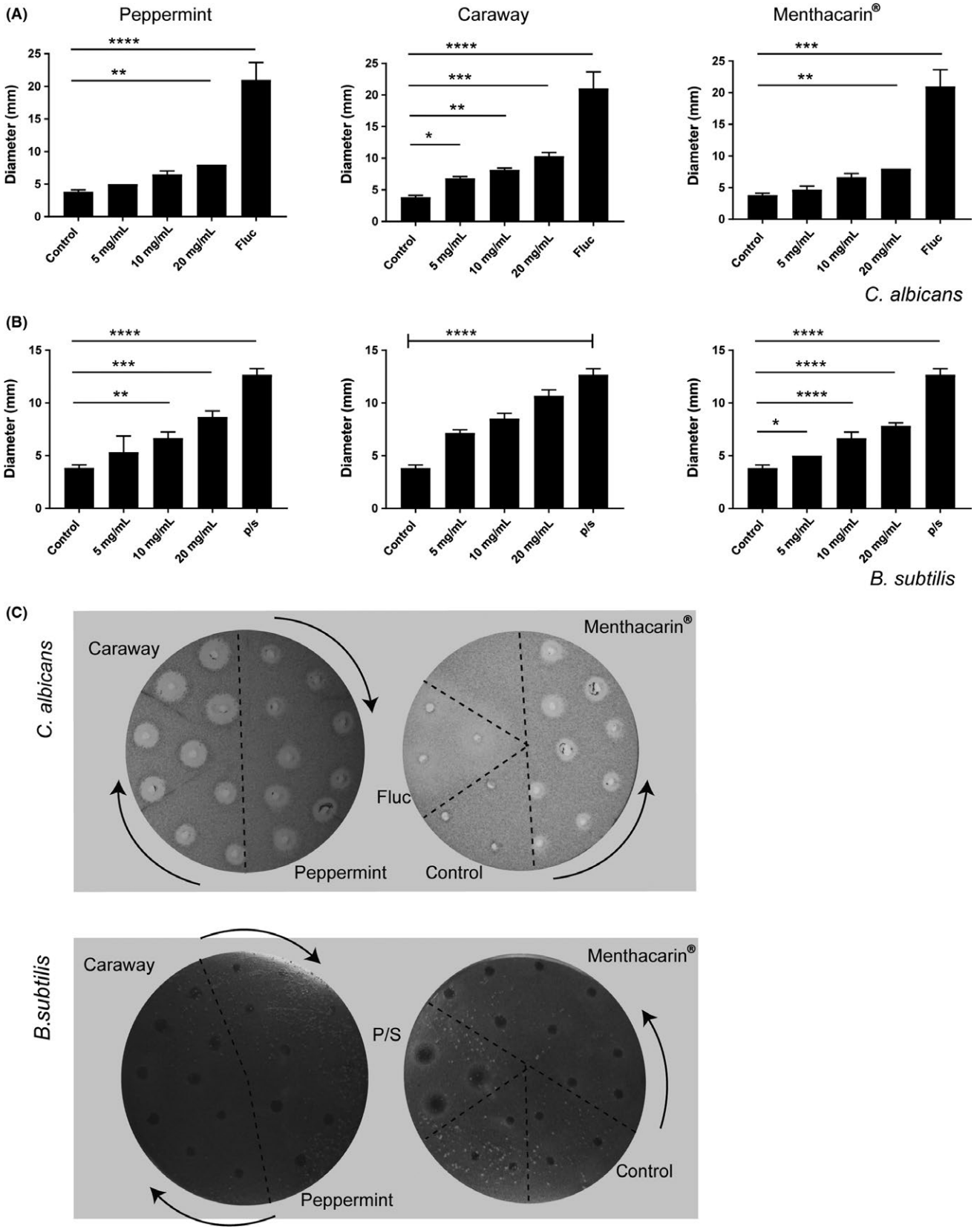

FIGURE 2 In vitro antifungal and antibacterial properties of Menthacarin® and its constituent's peppermint oil and caraway oil. Zones of inhibition in (A) *Candida albicans* seeded agar and (B) *B. subtilis* seeded agar, induced by peppermint oil (left panels), caraway oil (middle panels) and Menthacarin® (right panels). (C) Representative examples of the radial diffusion assay. Arrows indicate the starting direction of the concentration series (5, 10, and 20 mg mL^{-1}, all in triplicate). Positive controls were fluconazole (fluc) and penicillin-streptomycin (p/s). Data are mean ± SD, **P* < .05, ***P* < .01, ****P* < .001, *****P* < .00001 (n = 3/test condition)

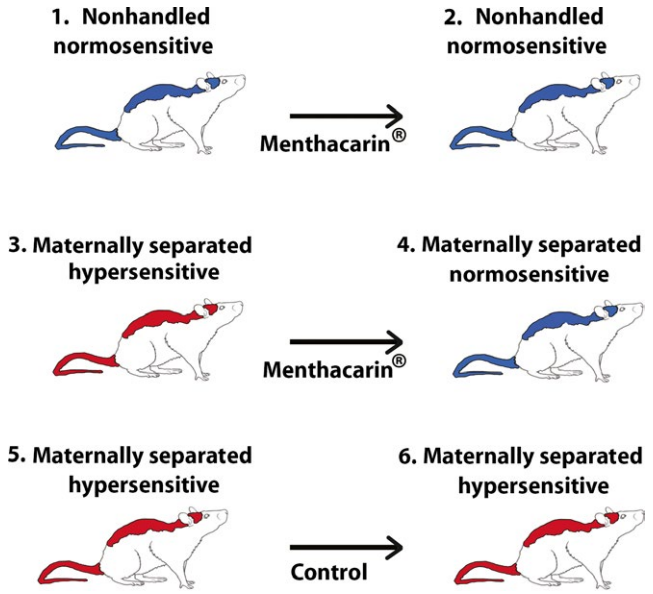

FIGURE 3 Schematic representation of color coded treatment groups used in mycobiome and microbiome analysis. For improved visualization of results depicted in Figures 4-7, normosensitive rats are shown in blue and hypersensitive rats in red. Sample groups 1 and 2 are non-handled rats before and after 75 mg kg^{-1} Menthacarin® treatment, groups 3 and 4 are maternally separated rats before and after 75 mg kg^{-1} Menthacarin® treatment, and groups 5 and 6 are maternally separated rats before and after control treatment

control treatment (groups 3, 5, and 6 respectively). The last branch included six of eight normally sensitive non-handled rats (group 1) that were sampled before Menthacarin® treatment. Thus, classical clustering confirmed results obtained with co-occurrence spectral analysis. Finally, our data set was visualized by non-metric multidimensional scaling to show the relation between predisposition (i.e. non-handled or maternally separated), treatment and mycobiome composition. This ordination technique revealed differential spatial patterns shown in Figure 5B. Non-Menthacarin®-treated maternal separated and non-handled rats localized to a broad and diffuse spatial area. After Menthacarin® treatment however, maternally separated and non-handled rats clustered, suggesting a high level of similarity between rats. In concert, our data suggest that Menthacarin® treatment is modulating the in vivo mycobiome and these changes associate with a normal response to colorectal distension.

3.4 | Maternal separation induced bacterial dysbiosis at multispecies level is not affected by Menthacarin® treatment

Although our results highlight the importance of intestinal fungi in IBS, a role for intestinal bacteria cannot be excluded. Menthacarin® showed in vitro bactericidal activity, and bacterial microbiome dysbiosis has been associated with IBS and visceral pain in the maternal separation model.[17,18] We therefore assessed bacterial microbiome composition and possible effects of Menthacarin® treatment, by next generation ribosomal DNA sequencing of the bacterial 16S V4 region. We generated 8925 unique taxonomic units, from which 174 different bacterial genera were taxonomically classified. Bacterial α-diversity was addressed using the relative abundance of unique taxonomic units. Except for a difference between non-handled Menthacarin® treated rats and non-treated maternally separated rats, we observed no significant differences in α-diversity (Figure 6A).

Classification of taxa at phylum level showed five dominant phyla. The relative abundance of none of these phyla differed when comparing pretreatment non-handled and pretreatment maternally separated rats (Figure 6B). However, Menthacarin® treated maternally separated rats had significantly lower Verrucomicrobia compared to all other (i.e. all hypersensitive) maternal separation rats (Figure 6C, group 4 vs groups 3, 5, and 6). Importantly, we only observed one species, *Akkermansia muciniphila*, within this phylum. This confirmed earlier reports on the limited intestinal representation of Verrucomicrobia.[37] We also observed changes in the distribution of Bifidobacterium, a genus that belongs to the Actinobacteria phylum. Maternal separated rats showed significantly lower relative abundance compared to non-handled rats and Menthacarin® treatment induced a significant increase in maternal separated rats (Figure 6D). Further analysis on phylum level showed that the relative abundance of Bacteroidetes in Menthacarin® treated non-handled rats was significantly lower compared to maternal separated rats before and after control. Compared to control treated maternal separated rats, Firmicutes were higher in Menthacarin® treated non-handled rats (data not shown).

Next, we performed co-occurrence spectral analyses to identify possible clustering of rats based on similarity at genus level (Figure 6E). We identified five main clusters, and most treatment groups were scattered across clusters. In consequence, the clustering pattern did not set apart pretreatment non-handled rats from pretreatment maternally separated rats. Seven of nine Menthacarin® treated maternally separated rats (group 4) clustered in the middle segment, but this segment also contained four control treated maternally separated rats (group 6). The absence of maternal separation induced microbiome dysbiosis and modulation thereof, suggested that bacterial dysbiosis was not critical for in vivo visceral sensitivity changes. Nevertheless, we decided to further address the bacterial microbiome with a classical clustering approach based on the Bray–Curtis dissimilarity index and the UPGMA algorithm (Figure 7A). For this evaluation we used unique sequences. At 0.6 similarity, the dendrogram divided into four branches and two separate Menthacarin®-treated non-handled (group 2) rats. The second branch from the right, contained two preMenthacarin® non-handled (group 1) rats. All other remaining non-handled rats (i.e. pre- and post-Menthacarin® group 1 and group 2 rats, n = 12) clustered in the third branch. The far right branch contained three pre-Menthacarin® treatment maternally separated (group 3) rats. All other maternally separated rats (n = 33) clustered, irrespective of treatment status, in the largest branch. Thus, according to this clustering method, the bacterial microbiome of non-handled rats differed from maternally separated rats, but Menthacarin®-treatment did not lead to a coherent microbiome shift in maternally separated rats.

Finally, we used non-metric multidimensional scaling to visualize the level of similarity between rats (Figure 7B). Pretreatment

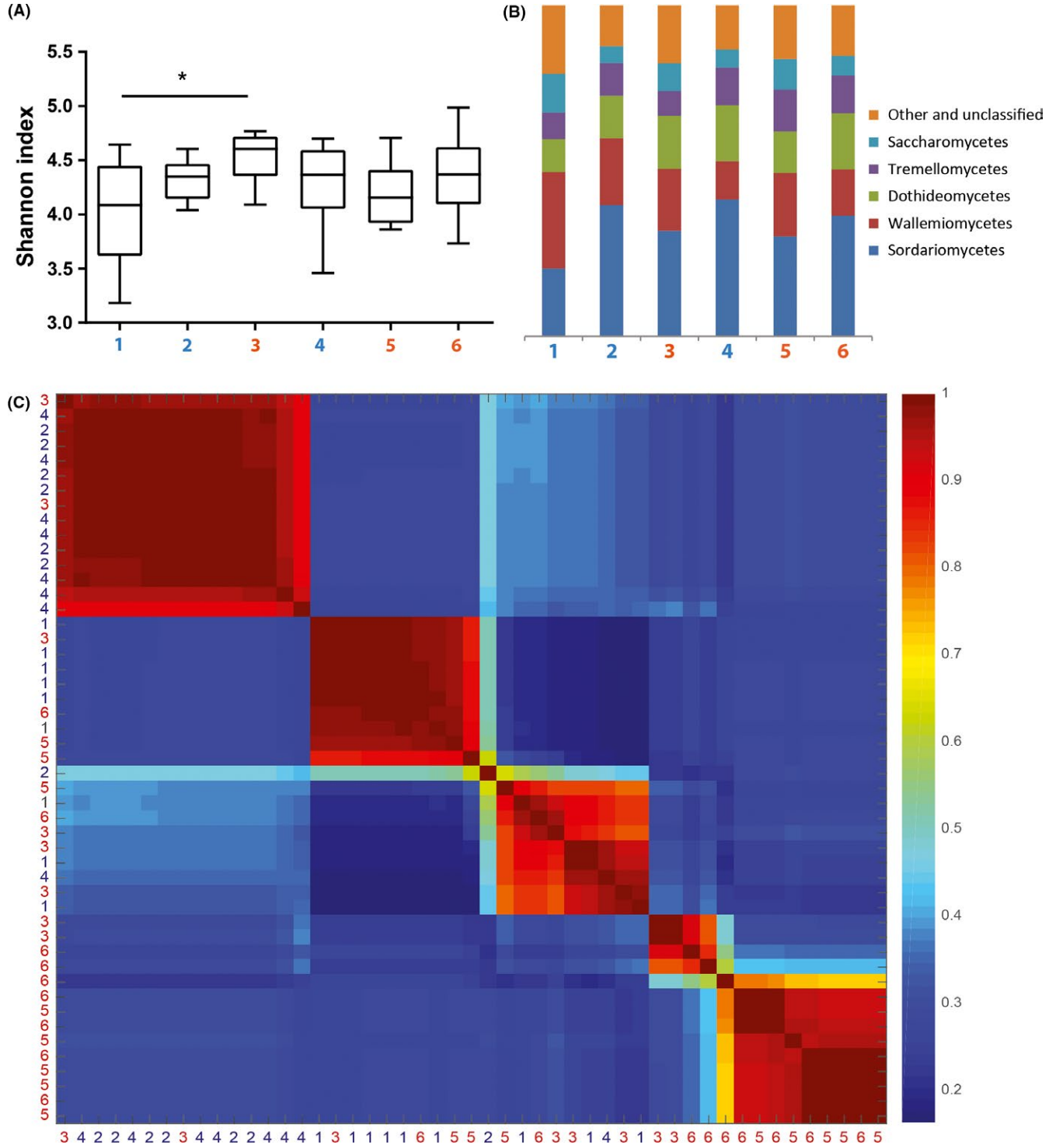

FIGURE 4 Maternal separation induced mycobiome dysbiosis and modulation thereof by Menthacarin® treatment. A, Box plot of Shannon diversity index based on species level showing median and 25-75% and 2.5-97.5% percentiles. *$P < .05$ (n = 7-9). B, Most abundant fungal classes present in the different sample groups. C, Co-occurrence matrix of clustering rats. Values range from 0.0 (dark blue for rats who never cluster) to 1.0 (dark red for rats that always cluster). Rat numbers on axes are symmetric and represent individual rats

maternally separated and pretreatment non-handled rats clustered in differential spatial areas, indicating marked differences between these groups. Menthacarin® treatment, however, did not separate the spatial positions of maternally separated rats before and after treatment. The rat positioned on the far left of the diagram did not show aberrant response to distension or lower read number but did have markedly different microbiome composition. Repeated calculations carried out without this particular sample did not result in major changes of the remaining scatter plot and left the conclusion unchanged (not shown). Taken together, this confirmed that bacterial dysbiosis is associated

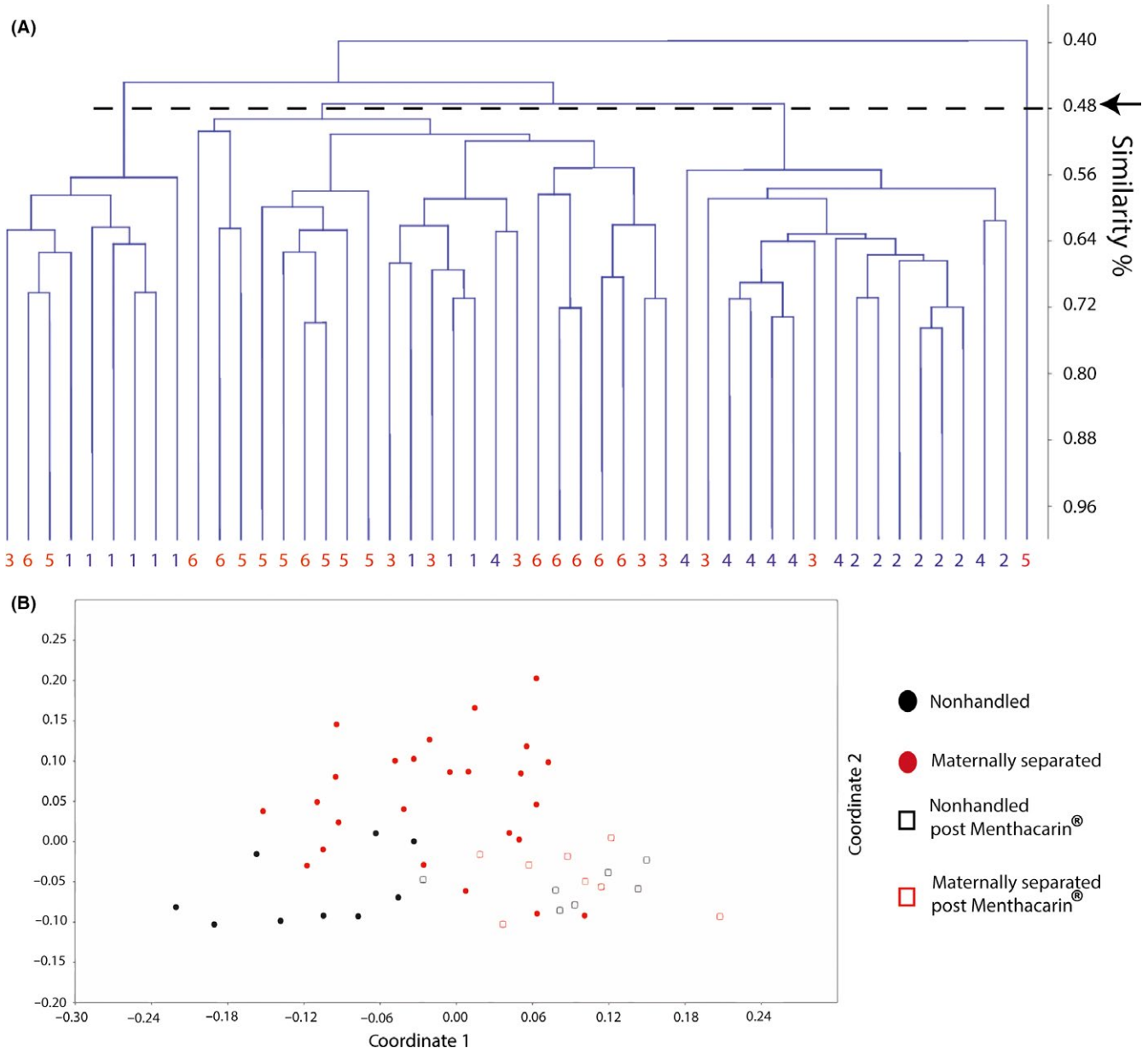

FIGURE 5 Mycobiome analysis using Bray–Curtis dissimilarity index and non-metric multidimensional scaling. A, Hierarchical clustering analysis using Bray–Curtis dissimilarity and UPGMA algorithm. Dotted line and arrow indicate 0.48% similarity. B, Non-metric multidimensional scaling plot based on the Bray–Curtis dissimilarity matrix (Stress: 0.191). Maternally separated rats are represented by red symbols, non-handled rats in black. All circles represent pretreatment rats, all open squares represent post-Menthacarin®-treatment rats

with maternal separation,[18,38] but Menthacarin® mediated reversal of poststress visceral hypersensitivity was not driven by major bacterial microbiome changes.

4 | DISCUSSION

Our previous research indicated that the use of antifungals could serve as a potential therapy for treatment of visceral hypersensitivity. We identified Menthacarin® as an antimicrobial and antifungal formulation. In the fungi dependent IBS-like rat model of maternal separation, 7 days Menthacarin® treatment reversed poststress visceral hypersensitivity. Fungal ITS and bacterial 16S ribosomal DNA barcoding assisted sequencing showed maternal separation induced changes in the myco- and microbiome. In maternal separated rats, Menthacarin®-induced reversal of visceral hypersensitivity was associated with marked changes in gut mycobiome but not microbiome composition.

A role for the intestinal microbiome is being investigated in the pathogenesis of a broad spectrum of diseases. The focus of most investigations has been on the bacteria, herewith ignoring the possible relevance of other components of the luminal ecosystem. More recent publications however, addressed the possible role of fungi in colitis models and IBD.[19-23] Our own investigations showed mycobiome

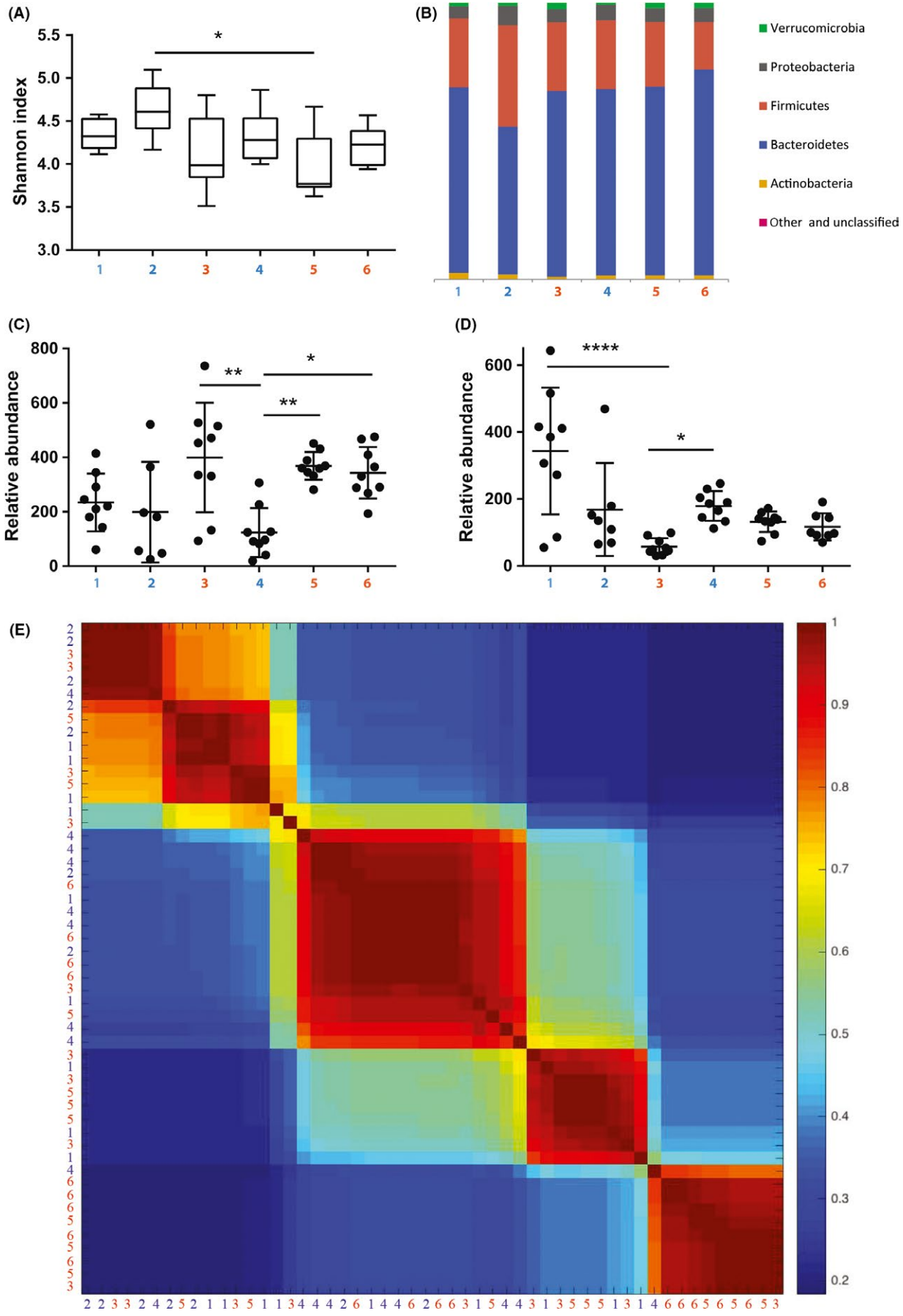
(A)
Shannon index
5.5
5.0
4.5
4.0
3.5
3.0
*
1 2 3 4 5 6
(B)
Verrucomicrobia
Proteobacteria
Firmicutes
Bacteroidetes
Actinobacteria
Other and unclassified
1 2 3 4 5 6
(C)
Relative abundance
800
600
400
200
0
**
**
*
1 2 3 4 5 6
(D)
Relative abundance
600
400
200
0
****
*
1 2 3 4 5 6
(E)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
2 2 3 3 2 4 2 5 2 1 1 3 5 1 1 3 4 4 4 2 6 1 4 4 6 2 6 6 3 1 5 4 4 3 1 3 5 5 5 1 3 1 4 6 6 6 5 6 5 6 5 3

FIGURE 6 Bacterial microbiome showed limited Menthacarin® induced changes. A, Shannon diversity index based on unique taxonomic units showing median and 25-75% and 2.5-97.5% percentiles. B, Most dominant phyla present in the different groups. C, Relative abundance of the Verrucomicrobia phylum and D, relative abundance of Bifidobacterium in treatment groups. E, Cooccurrence matrix of clustering rats. Values range from 0.0 (dark blue for rats who never cluster) to 1.0 (dark red for rats that always cluster). Rat numbers on axes are symmetric and represent individual rats. **P* < .05, ** *P* < .01 and *****P* < .0001 (n = 7-9)

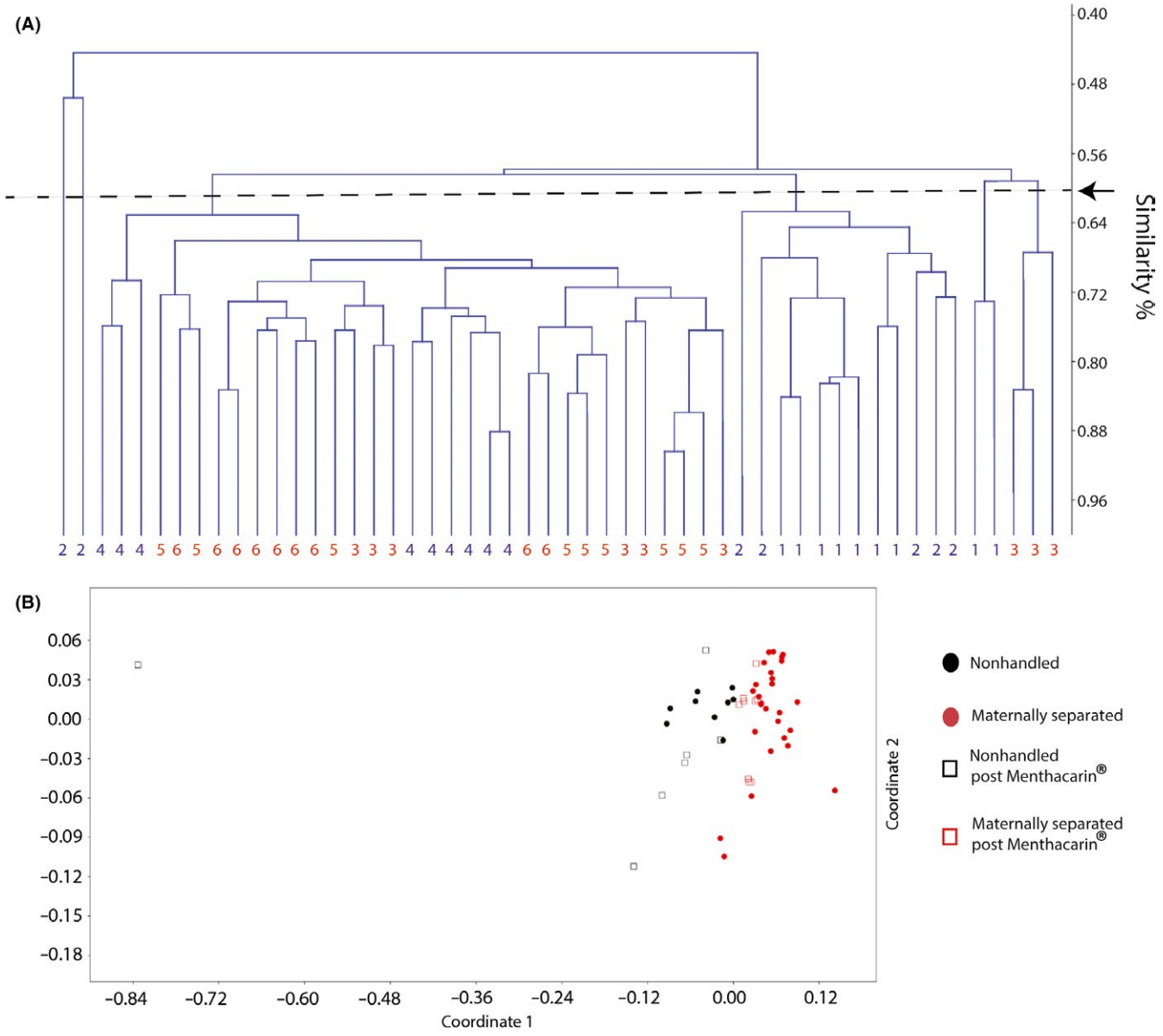

FIGURE 7 Bacterial microbiome analysis using Bray–Curtis dissimilarity index and non-metric multidimensional scaling. A, Hierarchical clustering analysis using Bray–Curtis dissimilarity and UPGMA algorithm. Dotted line and arrow indicate 0.60% similarity. B, Non-metric multidimensional scaling plot based on the Bray–Curtis dissimilarity matrix (Stress: 0.174). Maternal separated rats are represented by red symbols, non-handled rats in black. All circles represent pretreatment rats, all open squares represent post-Menthacarin®-treatment rats

dysbiosis in IBS patients, and similar results in the maternal separation model in rat. In this model, poststress visceral hypersensitivity was reversed by fungicide (fluconazole/nystatin) treatment. Subsequent fecal transfer experiments, from non-handled and maternally separated donor rats to these fungicide treated rats, indicated that only the maternal separation mycobiome conferred visceral hypersensitivity.[3] In the present study we confirmed maternal separation induced mycobiome dysbiosis. Moreover, Menthacarin®-mediated reversal of poststress visceral hypersensitivity associated with a shift in mycobiome composition. The resulting mycobiome highly resembled that of the Menthacarin® treated non-handled rats that also shifted away from their pretreatment mycobiome status. Non-handled rats remained normally sensitive after treatment, indicating the presence of a healthy mycobiome despite Menthacarin®-induced changes. Thus,

our results not only identified Menthacarin® as an effective treatment option for fungal-induced visceral hypersensitivity via mycobiome modulation, but also suggested that treatment of a healthy mycobiome will not lead to unfavorable side effects. The latter is relevant for future clinical trials because IBS is a heterogeneous disorder and, at this point, we are not capable of stratifying patients into those with or without fungal involvement in their abdominal pain complaints. In relation to clinical use, it should be mentioned that the same essential oil formulation was recently evaluated in a 4 weeks treatment protocol of patients with functional dyspepsia, where it relieved pain and discomfort.[12] During this clinical trial, Menthacarin® was administered two times daily at approximately 2 mg kg^{-1} dosages. In contrast, our maternally separated rats were treated for just 7 days and only received 1 daily gavage. Our relatively short and single dose administration strategy may explain why, despite adequate dose translation, rats treated with 25 and 75 mg kg^{-1} dosages but not with 12.5 mg kg^{-1} showed post-treatment reversal of visceral hypersensitivity. On the other hand, failure to reverse with the 12.5 mg kg^{-1} dosage may be due to the relatively high baseline sensitivity in this particular group of maternally separated rats, which didn't show significant increase upon WA stress. Since we only evaluated fecal microbiota of control and high dose Menthacarin® treated rats, we do not know whether the observed lack of reversal with low dose Menthacarin® associated with profound mycobiome changes or not. This is a limitation of the study.

When comparing baseline bacterial microbiome composition between non-handled and maternal separated rats, Bray–Curtis analysis, but not co-occurrence spectral analysis, showed bacterial microbiome dysbiosis. Moreover, Menthacarin®-induced reversal of poststress visceral hypersensitivity did not correlate with bacterial community changes at the multispecies level. This suggested that visceral hypersensitivity did not depend on bacterial dysbiosis and confirmed our earlier findings on the essential role of the gut mycobiome.[3] Nevertheless, changes observed in the relative abundance of *A. municiphila* may be important. In contrast with results obtained by Pusceddu et al[38], we did not observe a significant difference between non-handled and maternal separated rats. However, Menthacarin® treatment led to reduced relative presence of *A. municiphila* in maternally separated rats. Gobert et al[39] showed that this bacterium was over represented in constipation predominant IBS and additional experiments in mice suggested beneficial anti-inflammatory properties of *A. municiphila*. Therefore, future clinical trials should monitor long term effects on this potentially protective bacterium. Bifidobacteria are also considered to be health promoting members of the human intestinal microbiome and decreased levels were observed in IBS patients.[40,41] We found decreased relative abundance of Bifidobacteria upon maternal separation. Treatment with Menthacarin® correlated with a significant increase in this genus, suggesting that treatment created favorable conditions for these probiotic bacteria. Whether this was a direct Menthacarin® mediated effect or secondary to mycobiome modulation was not addressed in our investigations. Even so it can be envisaged that, despite the absence of major "multispecies level" menthacarin-induced changes in the bacterial microbiome and our earlier evidence on the relevance of gut fungi in the maternal separation model, microbiome changes such as those observed for bifido bacteria may also be relevant.

IBS prevalence rates are higher for women than for men.[1] Yet we choose to evaluate male rats only because the visceromotor response to distension fluctuates with the estrous cycle.[42] Although the different phases can be determined by microscopic evaluation of vaginal secretions, some problems remain to interfere with timing of a strict treatment protocol. Not only is the estrous cycle irregular in an approximate 30% of rats[43], maternally separated and non-handled rats also respond differently to colorectal distension in the distinct phases of the cycle.[44] Due to our focus on males, we cannot be sure that results on menthacarin-mediated reversal of visceral hypersensitivity and coincided mycobiome changes also apply to female rats. It should also be emphasized that the current investigations showed correlation instead of causal relationship between reversal of visceral hypersensitivity and mycobiome changes. Definitive proof for causality can be obtained by future fecal transfer experiments similar to those performed in our previous study.[3] In addition, we only focused on the gut myco- and microbiome, herewith neglecting other potentially relevant actions of Menthacarin®. Menthol is one of its active ingredients. It is known to desensitize, via TRPM8, the TRPV1 receptor which is relevant in visceral nociception in IBS and the maternal separation model.[10,11] Menthol is also a smooth muscle calcium channel antagonists that causes (similar to caraway)[45] muscle relaxation. This may be beneficial because dysmotility in IBS was shown to correlate with abdominal pain. Furthermore, menthol is known to have kappa opioid agonistic and serotonin receptor antagonistic activity which may both lead to analgesia.[6] Because we did not address these mechanisms, we cannot judge their relative weight for the observed reversal of visceral hypersensitivity. After 24 hours treatment in a recent clinical trial with peppermint oil, abdominal pain scores of the treatment group showed a significant 21% reduction from baseline vs 9% with placebo. At 28 days however, these numbers increased to 41% with peppermint oil vs 22% with placebo.[46] Possibly, results at the early time point reflected direct TRP channel and opioid receptor mediated effects only, whereas the doubled response at 28 days included more time demanding changes of the gut mycobiome. Unfortunately, the mycobiome nor any of the other possible mechanisms were addressed in this patient trial and the peppermint formulation lacked caraway oil, which was present in our investigations in rat. Irrespective, it can be argued that the possibility of synergistic analgesic activities make Menthacarin® an ideal candidate for IBS therapy. In the randomized placebo-controlled clinical trial in patients with functional dyspepsia that was mentioned earlier, the same peppermint- and caraway-oil-preparation was well-tolerated.[12] Finally, future use of this preparation would also satisfy patient preference for biological therapy.[47,48]

We previously suggested that gut mycobiome modulation can be an effective treatment for abdominal pain in IBS. The choice of antifungals in clinical practice is however limited and fungal resistance against these compounds is on the rise. Therefore, other approaches should be considered for a functional disorder like IBS. Our results indicate that Menthacarin® may be a safe and effective treatment option. In conclusion, we suggest that the propriety peppermint- and

caraway-oil-preparation Menthacarin® should be tested for the relief of abdominal pain complaints in IBS.

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DISCLOSURES

EK is an employee of Dr. Willmar Schwabe Pharmaceuticals. WJDJ has received consultancy fees from GlaxoSmithKline and receives research grant support from GlaxoSmithKline and Mead Johnsson Nutrition Pediatric Institute. All other authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

SB, OW, WJDJ, and RVDW designed experiments; EK supplied test compounds; SB, OW, and DMF conducted preclinical research and in vitro experiments; SB, EL, RCM, JS, TH, and FHJS performed ITS and 16S sequencing, analysis and statistics; SB and RVDW wrote the paper; all authors contributed to data analysis; all authors critically revised the manuscript for important intellectual content; all authors read and approved the final manuscript.

ORCID

R. M. van den Wijngaard http://orcid.org/0000-0002-0974-356X

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