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The impact of prebiotic supplementation in a triple-hit rat model of schizophrenia

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A B S T R A C T

Schizophrenia is associated with cognitive deficits and higher stress sensitivity, potentially related to gut-brain axis disturbances, partially due to dysbiosis. To ascertain this phenomenon, we aimed to evaluate these behavioral phenotypes in association with microbiota profile in the triple-hit Wisket rat model of schizophrenia. Furthermore, as a bidirectional approach, the effects of clozapine (CLO) and/or prebiotic (galactooligosaccharide, B-GOS) treatment were also investigated.

Male Wistar (control) and Wisket rats were treated for 3 weeks: CLO or its vehicle was administered intraperitoneally, while B-GOS or water was provided *ad libitum* in drinking bottle. The food-rewarded Ambitus test was used to assess cognition-related behaviors before and during the third week of the treatment. Afterwards, fecal samples were collected to analyse microbiota composition, and smooth muscle electromyography was performed to assess immobilization-induced stress response.

B-GOS monotreatment resulted in the highest improvement in cognition-related parameters in Wisket model rats; however, it never reached the performance of control animals. Wisket animals showed higher vulnerability to immobilization-induced stress condition. This group-difference disappeared by each pharmacological treatment, with the most prominent effect of the CLO + B-GOS combination treatment. β-diversity analysis revealed an overall compositional difference of fecal microbiota between treatment groups. Several taxa associated with schizophrenia-model or treatment were significantly correlated with behavioral parameters.

Consistent with clinical findings, cognitive impairment with increased stress sensitivity were highlighted in Wisket model rats. To our knowledge, this is the first study on B-GOS prebiotic in a triple-hit schizophrenia model, suggesting microbiome-targeted therapy may aid some schizophrenia-related symptoms.

1. Introduction

The functions of gut microbiota are not limited to digestion. They interact with the gut-brain axis, influencing brain development and behaviour. They also play a part in the development and activity of the hypothalamus-pituitary-adrenal (HPA) axis and interfere with drug metabolism (Sah et al., 2024; Trepka et al., 2025; Trisal et al., 2025). Gut microbiota communicate with the central nervous system via multiple pathways, including the enteric nervous system, neuroendocrine system, HPA axis, vagus nerve, or immune system. The composition of the microbiota can be influenced by several factors, including stress, dietary habits, prior infections and medication use, and it changes over time in response to environmental factors. Dysbiosis, or an imbalance in the composition of the microbiota, has been associated with various diseases directly related to the gastrointestinal (GI) tract, such as inflammatory and metabolic disorders, as well as with neuropsychiatric diseases including autism, anxiety, depression, Alzheimer's disease and schizophrenia (Lee et al., 2025). Gut dysbiosis can increase intestinal permeability and release proinflammatory cytokines that activate the HPA axis (Sah et al., 2024). Stress can further disrupt the gut microbiota,

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stimulating the HPA axis and enhancing the secretion of hormones such as cortisol, which are linked to neuropsychiatric disorders. It is also important to note that psychotropic medications may exacerbate this dysbiosis (Ait Chait et al., 2021).

In conclusion, both increased exposure and sensitivity to stress and gut dysbiosis also contribute to the pathophysiology of neuropsychiatric disorders. The behavioural improvements induced by targeting the gut microbiota (through pro- and/or prebiotic supplementation) clearly indicate these associations (Verma et al., 2024). However, some clinical studies investigating the effects of probiotic treatments containing *Bifidobacterium* and/or *Lactobacillus* on psychotic and non-psychotic symptoms in schizophrenia have produced conflicting results (Minichino et al., 2021; Romero-Ferreiro et al., 2025). While clinical findings indicate improved inflammatory and immune profiles in patients, the current evidence base does not clarify the beneficial role of probiotics in treating mental illness, except for major depression (Ribera et al., 2024). Prebiotics are commonly indigestible dietary components fermented in the colon by resident bacteria, primarily by *Bifidobacteria* and *Lactobacilli,* to produce short-chain fatty acids (SCFAs) that have multiple beneficial physiological effects (Fusco et al., 2023). They can alleviate gut dysbiosis by inducing the proliferation of beneficial intestinal bacteria, and improve anxiety, cognition, neural activity, and stress response (Ait Chait et al., 2021; Liu et al., 2021). Recently, a placebo-controlled study provided proof-of-concept that prebiotic Bimuno™ galactooligosaccharide (B-GOS) supplementation improves cognitive flexibility in psychosis (Kao et al., 2019).

Regarding the preclinical findings, inulin administration, a natural fructan with prebiotic properties, improved the locomotor hypoactivity, anxiety disorders and depressive behaviours of mice with an MK801-induced schizophrenia model, as well as their impaired learning and spatial recognition memory (Guo et al., 2021). A recent study demonstrated cognitive improvement and enhanced gastrointestinal function, as well as beneficial modulation of the gut microbiota and related metabolic pathways, following chronic prebiotic combination treatment in an mGlu5 knockout mouse model (Gubert et al., 2025). While single-hit schizophrenia models reproduce patterns of either positive, negative and cognitive symptoms, the triple-hit Wisket rat model — combining postweaning isolation rearing, ketamine administration, and selective breeding — better reflects the multifactorial etiology of the disorder (Percelay et al., 2024; Petrovszki et al., 2013). To date, the effects of prebiotic interventions have not been investigated in such complex models. Based on existing evidence, prebiotics may attenuate stress induced by isolation rearing partially due to reduced corticosterone release (Burokas et al., 2017; O'Mahony et al., 2020) and ameliorate ketamine-associated behavioural deficits by modulating the tryptophan–kynurenine pathway and restoring NMDAR hypofunction through microbiota-derived acetate production (Gronier et al., 2018; Savignac et al., 2013; Singh et al., 2016; Xu et al., 2025).

Our previous study revealed dysbiosis in the Wisket rat model of schizophrenia in association with behavioural phenotype (Plesz et al., 2025). The present study aimed to investigate whether dysbiosis is linked to increased stress sensitivity by measuring stress-induced GI myoelectric changes, which correlate with plasma cortisol levels and smooth muscle contraction (Pribék et al., 2021; Szűcs et al., 2018; Szucs et al., 2016). In view of frequent comorbidity of GI and neuropsychiatric disorders, as well as the impact of antipsychotics on smooth muscle activity, we examined the effect of B-GOS and/or antipsychotic (clozapine, CLO) treatment on dysbiosis, cognitive function, and stress sensitivity. While studies have highlighted antipsychotic-induced dysbiosis and the benefits of prebiotics and probiotics, little is known about the microbiome-drug interactions in antipsychotic efficacy. To our knowledge, this is the first study on B-GOS in a triple-hit schizophrenia model, suggesting that microbiome-targeted therapy may aid schizophrenia-like phenotype.

2. Experimental procedures

2.1. Ethical considerations

All efforts were made to minimise animal suffering and reduce the number of animals used in the study. The experiments were performed with the approval of the Hungarian Ethical Committee for Animal Research (registration number: XIV/1421/2023 and XIII/72/2020). The study complied with the ARRIVE and followed the guidelines established by the Government of Hungary and EU Directive 2010/63EU for animal experiments.

2.2. Wisket rat model of schizophrenia

The Wisket rat model of schizophrenia was developed by combining post-weaning isolation rearing for 4 weeks (environmental hit #1), NMDA-antagonist ketamine treatment (pharmacological hit #2; 30 mg/kg intraperitoneally for 3 weeks from 4 weeks of age) and selective breeding based on behavioural phenotype (genetic hit #3) (Fig. 1) (Petrovszki et al., 2013). During resocialisation at 8 weeks of age, the animals were pair-housed (matched by sex and group) as part of the breeding process. For male subjects, this housing condition was important to minimise the effects of the cage on faecal microbiota composition. Control Wistar rats were socially reared and not treated with ketamine, and the Wistar and Wisket trait animals were not littermates; they were simply raised together and matched by age.

2.3. Experimental paradigm

Male Wistar and Wisket model rats were involved in the study (n = 6-8/group). Due to the large number of treatment groups, modest group sizes were applied to comply with the ethical considerations of 3Rs, and also similar to other studies involving behavioural-pharmacological tests or microbiome analysis (Kao et al., 2018b; Yang et al., 2018). The ambient temperature was maintained at 21 (±2 °C) with a 12 h light/dark cycle. Food and water were available *ad libitum*, except during the Ambitus tests and smooth muscle electromyography (SMEMG) recordings. The animals were food-restricted for two days before Ambitus testing, and moderate food restriction (approx. 40% of the daily requirement) was maintained throughout the 3-day long posttreatment testing period. Food and water were also withdrawn 2 h before and during the SMEMG recordings. Behavioural tests were performed between 8 a.m. and 4 p.m. The rats' body weight was carefully controlled throughout the experiment.

On week 10, all animals underwent the 1-day long pretreatment Ambitus test (see 2.3.1; pretreatment parameters; Fig. 1). Pharmacological treatment began in week 11 and continued for three weeks (weeks 11–13). CLO (2.5 mg/kg, Leponex®, Mylan EPD Kft., Budapest, Hungary) was daily injected intraperitoneally (i.p.), while B-GOS (15 g/L, Bimuno®, GAL SynergyTech Zrt., Budapest, Hungary) was provided *ad libitum* in a drinking bottle diluted with tap water. To ensure that the injection-induced physical stress affected all animals equally, the vehicle of clozapine (ethanol:Macrogol 400:sterile water for injection; 1:5:4) was injected to B-GOS-monotreated animals. The animals were thus assigned to four treatment groups in both the control and Wisket traits: CLO i.p. + tap water, vehicle i.p. + B-GOS, CLO i.p. + B-GOS, and vehicle i.p. + tap water. Fresh solutions were provided three times a week. The doses and the treatment duration were based on the literature (Gray et al., 2009; Yang et al., 2018). Fluid and food intake were recorded three times a week. Body weight was recorded and relative food and fluid consumption and weight gain were analyzed only during unrestricted periods (weeks 11-12). During the third week of the pharmacological treatment, the animals underwent a 3-day long posttreatment Ambitus test. Thereafter, the rats were individually transferred to disinfected cages to obtain fresh fecal pellets from spontaneous defecation. These were collected into a sterile Eppendorf tube and stored at

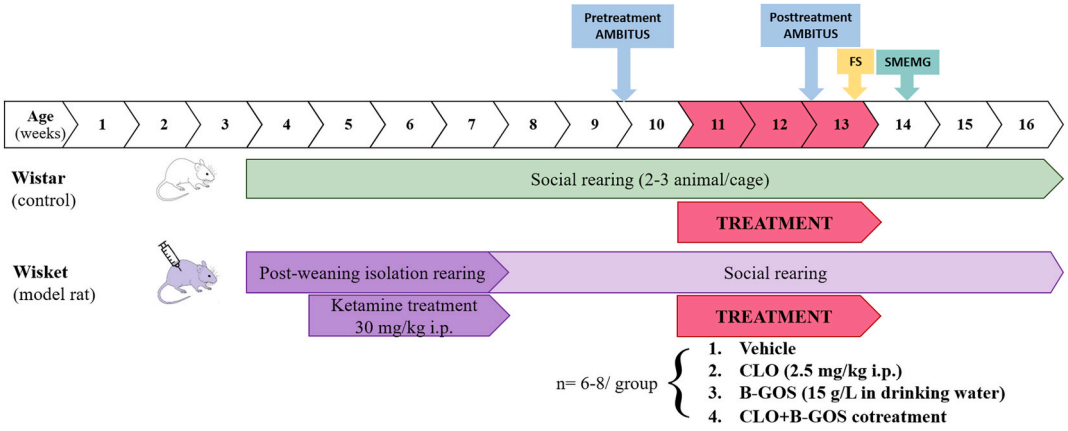


Fig. 1. Experimental paradigm. Abbreviations: FS – fecal sampling; SMEMG – smooth muscle electromyography; B-GOS – galactooligosaccharide; CLO – clozapine; VEH – vehicle; i.p. – intraperitoneal.

−70 °C until microbiota analysis. On week 14, following cessation of treatment, subcutaneous electrodes were implanted above the GI tract for SMEMG recording (see 2.3.2). At the end of the experiments, the animals were terminated by carbon dioxide inhalation.

2.3.1. *Food-rewarded ambitus test*

The Ambitus apparatus is a rectangular corridor with side-boxes (www.deakdelta.hu) that was used to assess the basal locomotor and exploratory activities as well as cognition-related parameters in rats (Supplementary Fig. S1). The animals were allowed to explore the corridor and collect food rewards (puffed rice) from side-boxes for 5 min. Each rats performed two sessions (two trials/session, 2 min apart) per day, one in the morning, and another 3 h later (Supplementary Fig. S2). The mean of the two trials per session was further analyzed, except for working memory calculations, where the data from specific trials were evaluated. Different tasks with altered baiting patterns were used: for Task 1, all of the internal and external boxes were baited (16 rewards); for Tasks 2 and 3 only the internal or external boxes were baited (8-8 rewards, respectively). The analyzed parameters are indicated in Table 1.

2.3.2. *Electromyographic measurements*

The rats were anaesthetised by isoflurane inhalation, then a bipolar disk electrode pair (SEN-15-2; MSB-MET Ltd., Balatonfüred, Hungary) was fixed subcutaneously 1 cm right from the midline above the GI tract (Pribék et al., 2021). The sensor cable was led subcutaneously, with the terminal being led out through the skin of the neck. The abdominal and cervical incision surfaces were then closed with surgical sutures and staples, respectively.

Myoelectric recordings were performed on the day after the surgery.

Table 1
Cognition-related parameters measured and/or calculated in the Ambitus test.

Parameter	Unit	Definition and Calculation
Omission error	Ratio	The ratio of food rewards explored but not collected: $1 - \frac{\text{number of collected food rewards}}{\text{number of baited box visits}}$
Motivation	Ratio (%)	A ratio indicating the animal's motivation to collect food rewards, predicting cognitive parameters: $\frac{\text{number of collected rewards} \times \text{cut} - \text{off time [300 s]}}{\text{maximum number of rewards} \times \text{task completition time [s]}} \times 100$
Working memory index	Ratio (%)	A ratio that describes the visuo-spatial short-term memory: $\frac{\text{number of food rewards eaten}}{\text{number of rewarded visits and revisits into previously rewarded boxes until task completititon}} \times 100$

The animals were placed individually in cages with high-pitched walls, each featuring a transparent front. The animals were not restricted in their movements for 120 min while basal myoelectric activity was recorded. The rats were then anaesthetised with 1.5% isoflurane by inhalation until they were placed and fixed in a prone position on a glass plate with strong sticky belts, so that they were unable to move or turn around. After full awakening (3–5 min), the GI activity was recorded again for 60 min under this restrained condition (stress response) (Supplementary Fig. S3).

Myoelectric signals were recorded and analyzed by an online computer and amplifier system by the S.P.E.L. Advanced ISOSYS Data Acquisition System (MSB-MET Ltd., Balatonfüred, Hungary). Five animals (out of 56) were excluded from the analysis of SMEMG measurements due to technical issues. The SMEMG recordings were filtered within the frequency ranges of 3–5, 20–25 and 1–3 cpm (cycles per minute), which are indicative of stomach, ileum and cecum activity, respectively. To eliminate differences due to phase lengths (120 vs. 60 min), the myoelectric activity at rest was evaluated in two phases (1-60 and 60-120 min). As these showed no differences, the basal maximum power spectrum density (PsD_{max}) values were calculated as their average. During the evaluation, the PsD_{max} of basal activity was compared to activities during the stress period. The change in PsD_{max} perfectly reflects the change in the contractions of the smooth muscle (Szucs et al., 2016).

2.3.3. *DNA isolation, 16S rRNA gene library preparation and MiSeq sequencing*

DNA extraction, 16S rRNA gene amplification, and sequencing were carried out as described previously (Plesz et al., 2025) with the following modifications: bacterial DNA was isolated using the ZymoBIOMICS DNA Kit (Zymo Research, Irvine, CA, USA), and purified with sparQ PureMag Beads (Quantabio, Beverly, MA, USA). Library quality control was performed using the Agilent DNA 7500 Kit (Agilent Technologies, Waldbronn, Germany). All other procedures followed the previously published protocol.

Following MiSeq sequencing, essentially the bioinformatic analysis was carried out as described by Mansour et al. (2020), and also detailed in our previous work (Plesz et al., 2025).

2.3.4. *Bacterial quantification by qRT-PCR*

Specific primers were designed in order to assess the total number of bacteria in the feces (Supplementary Table S1). Briefly, bacterial 16S rRNA gene sequences were obtained from the European Nucleotide Archive and aligned in UGENE (Okonechnikov et al., 2012) to identify potential specific primers. The final primers were selected based on testing them in SILVA (Glöckner et al., 2017) and Ribosomal Database

Project (RDP) (Cole et al., 2009) rRNA gene databases. Primers were synthesized commercially (Integrated DNA Technologies, IA, USA). Bacterial DNA was extracted from 50 to 100 mg fecal samples by ZymoBIOMICS DNA Miniprep Kit (Zymo Research, USA). Then, quantitative PCR (qPCR) was performed on a LightCycler® 480 II instrument (Roche, Germany) using a SensiFAST SYBR No-ROX Kit (Bioline, London, UK). A standard curve ranging from 10^5 to 10^9 CFU/ml was produced from amplicons derived from the control strain *E. coli* DH5α (ATCC PTA-1798) to convert cycle threshold (Ct) values into bacterial quantity (log CFU/gram sample).

2.4. Statistical methods

The Ambitus datasets were analyzed using a repeated-measures ANOVA with group and treatment as the categorical factors. Factorial ANOVA was used for data obtained without repetition (weight gain, average fluid intake and B-GOS intake). The LSD post hoc test was performed and a probability of $p < 0.05$ was considered statistically significant (Statistica software, version 14.0.1.25, TIBCO Software Inc., USA).

For the SMEMG evaluation, a paired *t*-test was used to describe stress-induced alterations in PsD_{max} values compared to the corresponding basal myoelectric activity (self-control), and an unpaired *t*-test was used to statistically compare pharmacological interventions, with a significance level defined as $p < 0.05$ (Prism 10.4, GraphPad Software, LLC, USA). Alpha diversities were quantified using the Shannon index. Taxa with at least 50 reads were considered positive; the rest were discarded from the downstream analysis. Differential abundance tests were conducted using the Wilcoxon rank-sum test, with the Benjamini–Hochberg method applied to estimate the false discovery rate (FDR) at a threshold of 0.05. Permutational multivariate analysis of variance (PERMANOVA) was performed to test for significant differences between the treatment groups.

A correlation analysis of behavioural parameters and relative bacterial abundances was conducted as described previously (Plesz et al., 2025). In brief, Spearman's rank correlation coefficients (Rho-values) were calculated for each possible pair of behavioural parameters and bacterial abundances at four distinct taxonomic levels (phylum, class, family and genus), including the data of all samples. The corresponding adjusted p-values (q-values) were obtained by applying the Benjamini–Hochberg procedure. For correlation analysis a significance level of 0.05 was chosen. Heatmaps with accompanying dendrograms were generated to visualize the results of the correlation analysis (Hutka et al., 2021; Lázár et al., 2021).

A microbiota-behaviour correlation network, a bipartite graph, was constructed, based on significant correlations (unadjusted $p < 0.05$) between behavioural parameters and the relative abundance of bacterial genera. This network was visualised using the Cytoscape network analysis platform (version 3.10.3) (Shannon et al., 2003) by applying a relative entropy optimisation-based algorithm (EntOptLayout plugin; version 2.1) (Ágg et al., 2019). Correlation analysis and network construction were performed using the R programming environment (version 4.3.3) (R Core Team, 2024).

3. Results

3.1. Schizophrenia-like behavioural changes in Wisket model rats

Consistent with our previous findings (Horvath et al., 2021; Plesz et al., 2025), a significant group effect was revealed for each of the parameters investigated. Thus, Wisket animals exhibited reduced exploration of both baited and non-baited boxes, along with higher omission errors, motivational and working memory deficits in the pretreatment Ambitus test (Supplementary Table S2). None of the parameters differed significantly between the later, randomly selected treatment groups.

3.2. Weight gain and B-GOS intake

Regarding weight gain (% increase) during the 1st and 2nd week of the pharmacological treatment, when food was freely available for the animals, factorial ANOVA revealed a significant effect of treatment ($F_{(3,48)} = 3.78$; $p < 0.05$). However, the analysis did not reveal any significant effects of group or group-treatment interaction. Nevertheless, the post hoc analysis showed significantly higher weight gain in the Wisket CLO-treated group (9.68 ± 0.72%) than in the vehicle-treated (4.19 ± 0.95%) and combination-treated (5.53 ± 0.85%) counterparts despite similar food intake. This suggests the presence of some antipsychotic-induced metabolic effects.

The average fluid intake was significantly lower in Wistar model rats ($F_{(1,54)} = 24.35$, $p < 0.0001$) than in controls (37.65 ± 1.89 vs. 49.70 ± 1.58 mL/bw kg/day). Factorial ANOVA regarding B-GOS intake revealed a significant group effect ($F_{(1,24)} = 7.79$; $p < 0.05$) and a significant group-by-treatment interaction ($F_{(1,24)} = 5.58$; $p < 0.05$), with significantly lower B-GOS intake in the Wisket group (562.50 ± 35.66 mg/bw kg/day) than in the control group (785.00 ± 37.69 mg/bw kg/day) in the case of B-GOS monotherapy. However, this group difference was not observed in the CLO + B-GOS co-treatment group (669.59 ± 54.06 vs. 661.05 ± 29.12 mg/bw kg/day), as a decrease in fluid consumption was also measured in the control group.

3.3. B-GOS treatment is superior to CLO treatment in improving schizophrenia-associated behavioural deficits

Since repeated-measures ANOVA revealed significant group differences regarding the investigated posttreatment parameters, with a kind of floor effect in the Wisket model rats, separate graphs were prepared to visualize the pharmacological effects in the control and Wisket groups.

3.3.1. Exploration

Significant effects of group, treatment, and time were revealed for both the baited ($F_{(1,48)} = 85.09$, $p < 0.0001$, $F_{(3,48)} = 4.43$, $p < 0.01$ and $F_{(5,240)} = 4.43$, $p < 0.01$) and non-baited ($F_{(1,26)} = 85.98$, $p < 0.001$, $F_{(3,26)} = 7.77$, $p < 0.001$ and $F_{(4,104)} = 4.54$, $p < 0.01$) box explorations, with significantly reduced exploration in Wisket animals that never reached the control levels (Supplementary Fig. S4). Post hoc analysis showed significant improvement with CLO or B-GOS monotreatments compared to vehicle and CLO + B-GOS treatment in the controls, with a modest B-GOS-induced improvement in the Wisket animals (Supplementary Fig. S4).

3.3.2. Omission error

For omission errors significant effects of group ($F_{(1,48)} = 48.42$, $p < 0.0001$), time ($F_{(5,240)} = 10.45$, $p < 0.0001$), and group and time interactions ($F_{(5,240)} = 6.92$, $p < 0.0001$) were observed. While control animals showed negligible omissions, Wisket rats displayed higher values, indicating a failure of reward consumption (Fig. 2A and B). Post hoc comparisons revealed significant improvement in all Wisket groups compared to their vehicle-treated counterparts. This led to the decline in the difference between the control and Wisket groups from session 3 onwards following B-GOS and CLO + B-GOS combination treatment.

3.3.3. Motivation

Repeated-measures ANOVA revealed significant effect of group ($F_{(1,48)} = 107.57$, $p < 0.0001$), treatment ($F_{(3,48)} = 4.38$, $p < 0.01$), and time ($F_{(5,240)} = 47.07$, $p < 0.0001$) regarding the motivation index, which highly predicts cognitive performance (Fig. 2C and D). Furthermore, the interactions between group and treatment ($F_{(3,48)} = 3.14$, $p < 0.05$), group and time ($F_{(5,240)} = 19.43$, $p < 0.0001$), treatment and time ($F_{(15,240)} = 2.99$, $p < 0.001$), and group and treatment and time ($F_{(15,240)} = 1.90$, $p < 0.05$) were also significant. The greatest improvement was seen in animals treated with B-GOS in the control group across the sessions. Post hoc analysis showed that there was a

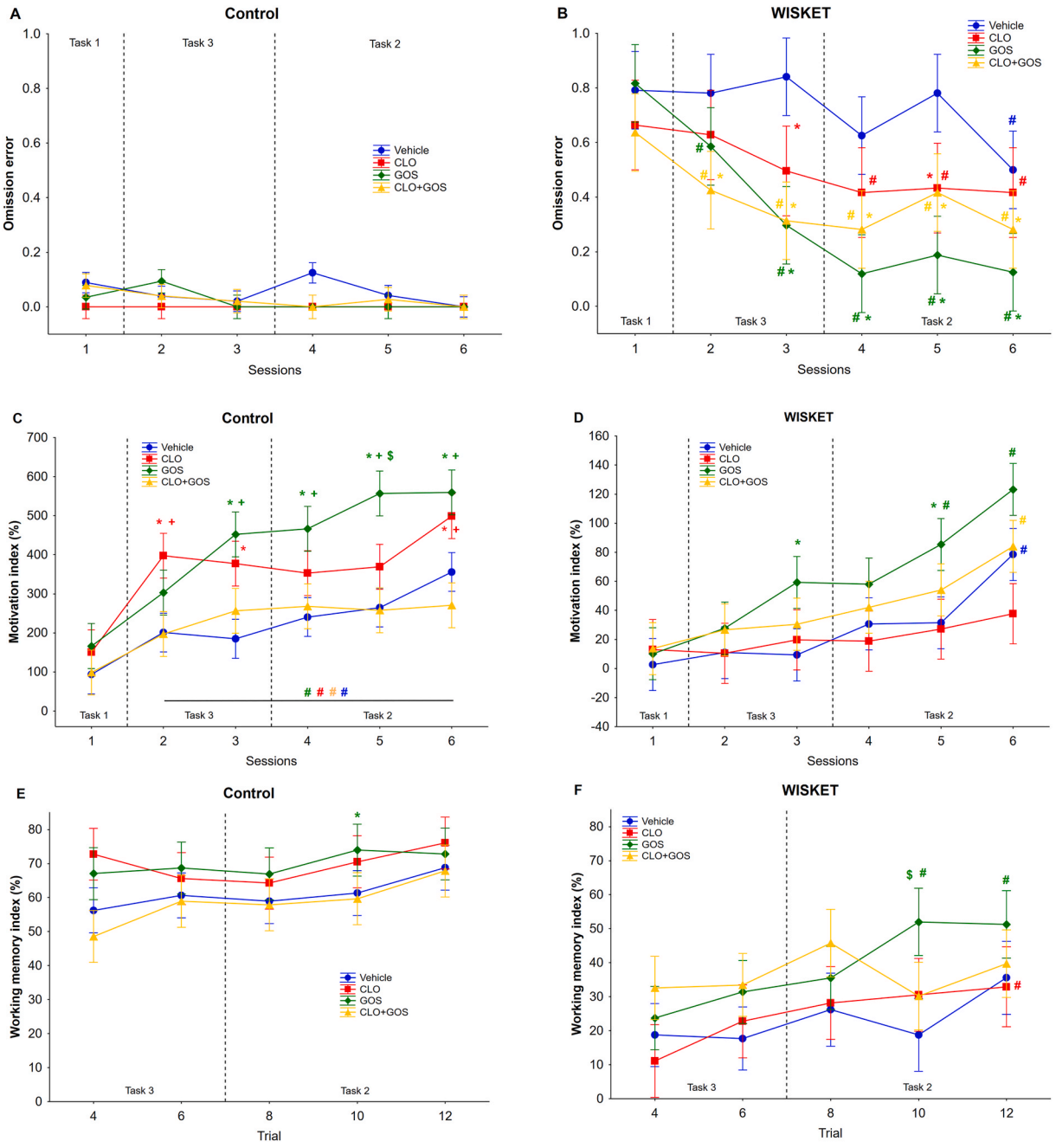


Fig. 2. Number of omission errors (A, B) and the motivation index (C, D) by sessions, and the working memory index (E, F) by trials for the control (left) and Wisket (right) groups. Vertical dashed lines indicate the switch to another task during the Ambitus paradigm. Data are presented as mean ± SEM. The symbols indicate significant ($p < 0.05$, repeated-measures ANOVA) differences compared to vehicle treatment (*), between mono- and combination treatment (+), compared to session 1 values or to trial values (#), and between CLO and B-GOS monotreatments ($).

session-related improvement with CLO and B-GOS monotreatments outperforming the vehicle-treated controls. B-GOS seemed to be more effective than CLO, whereas the CLO + B-GOS combination showed no significant benefit (Fig. 2C). In Wisket model rats, post hoc analysis indicated significant, albeit delayed, improvement by session, except in the CLO group (Fig. 2D). However, none of the treatments restored performance to control levels. Post hoc comparisons did not reveal differences between the treatment groups, although a tendency towards greater improvement was observed in the B-GOS-treated group, similar to their control counterparts despite lower B-GOS intake (section 3.2).

3.3.4. *Working memory*

Since different tasks were applied and repeated during the posttreatment Ambitus testing on the same day and/or on consecutive days (Supplementary Fig. S2), we could also calculate the working memory index to describe the short-term memory (Table 1). The significant effects of group ($F_{(1,40)} = 36.41$, $p < 0.0001$) and time ($F_{(4,160)} = 2.98$, $p < 0.05$) indicate short-term memory deficit in the Wisket group, which improved with practice, as revealed by the post hoc comparison in the CLO and B-GOS treated groups (Fig. 2E and F). The pharmacological treatments induced an enhancement in working memory, resulting in the disappearance of significant differences between the control and Wisket counterparts, with the exception of the CLO-treated group.

3.4. In vivo SMEMG results

3.4.1. Basal myoelectric activity and stress-response associated with shizophrenia

No significant differences in basal PsD_{max} values were revealed between the vehicle-treated control and Wisket groups in any of the GI tract regions were revealed, thus similar basal myoelectric activities were recorded (Fig. 3).

The immobilization-induced stress condition resulted in significant increase in the PsD_{max} values both in the vehicle-treated control and Wisket groups in the stomach and small intestine (Fig. 3A and B). Regarding the cecum, significant increase in PsD_{max} value was only observed in the Wisket group (Fig. 3C). It should also be noted that there was a large variance in stress-related PsD_{max} values indicating the high individual variability of stress sensitivity within a group. Significantly higher PsD_{max} values were measured in vehicle-treated Wisket group than in their control counterparts in the stomach and cecum, indicating the higher stress sensitivity of the model rats.

3.4.2. Pharmacological effects on basal myoelectric activities

Regarding the basal PsD_{max} values, a tendency towards decreased myoelectric activity was observed in each of the pharmacologically treated groups compared to their vehicle-treated counterparts. This reached a significant level in the cecum and stomach of CLO-treated control rats and CLO + B-GOS-treated Wisket model rats (Fig. 3A–C).

3.4.3. Pharmacological effects on stress response

In the stomach and cecum, the PsD_{max} values remained significantly elevated in response to stress compared to their respective basal values in both the control and Wisket groups following each pharmacological treatment (Fig. 3A–C). However, the significant group difference in the stress response, that was observed between vehicle-treated controls and Wisket animals, disappeared with each pharmacological treatment. Furthermore, significantly lower myoelectric activity was measured in the CLO + B-GOS cotreated Wisket group compared to their vehicle-treated counterparts, indicating the advantage of combination therapy over monotreatments.

Regarding myoelectic activity in the small intestine the statistical analysis revealed no effects of the pharmacological treatments on the stress response either in the control or in the Wisket group. Thus the PsD_{max} values in stress remained significantly higher than the corresponding basal values (Fig. 3B). Furthermore, no significant differences in the stress response occurred between the pharmacologically and vehicle-treated groups.

3.5. Fecal microbiota changes by pharmacological treatment

The qRT-PCR measurement did not reveal any significant differences between groups regarding total bacterial amounts (Fig. 4A). Therefore, relative abundance data were used for statistical analysis. Although alpha diversity metrics did not differ between groups at any taxonomic level, beta diversity analyses revealed significant differences in microbial community structure associated with the model and treatment (Table 2).

Comparing the microbiota composition of vehicle-treated control and Wisket rats confirmed our previous finding that the common gut microbiota is significantly altered in rats exhibiting schizophrenia phenotype (Plesz et al., 2025). This dysbiosis was characterised by a significant decrease in the relative abundance of several genera belonging to the *Lactobacillaceae*, *Bacillaceae*, *Bifidobacteriaceae*, *Saccharimonadaceae*, *Enterococcaceae*, *Staphylococcaceae* and *Streptococcaceae* families. This compositional change resulted in a significant decrease in the *Firmicutes/Bacteroidetes* ratio in Wisket rats (1.3 ± 0.15 vs. 1.6 ± 0.18) (Table 2 and Fig. 4B). In contrast, significantly increased abundance of the genus *Erysipelotrichaceae-UCG-003* was evident in Wisket animals.

In terms of bacterial changes caused by pharmacological treatments, both CLO and B-GOS only had a modest effect on the composition of the gut microbiota in control rats. Notably, the affected taxa differed from those affected by the Wisket phenotype. For example, *Roseburia* levels increased and *Lachnospiraceae-UCG-008* levels decreased in CLO- and CLO + B-GOS-treated control rats, suggesting that CLO notably impacts these genera. CLO also increased *Dubosiella*, *Parasutterella* and *Phascolarctobacterium*, while B-GOS increased *Prevotella-7* and *UCG-008*.

In Wisket rats, however, treatment with either CLO or B-GOS improved Wisket-associated dysbiosis, resulting in the partial normalisation of levels of *Streptococcus*, *Staphylococcus*, *Pediococcus*, *Enterococcus*, *Bacillus*, and several *Lactobacillaceae* genera. These changes led to an increased abundance of *Firmicutes* and an improved *Firmicutes/Bacteroidetes* ratio (Wisket CLO: 1.6 ± 0.05; Wisket B-GOS: 1.6 ± 0.12; Wisket CLO + B-GOS: 1.5 ± 0.11). Apart from these effects, the significant decrease in *Lachnospiraceae-UCG-008* observed in control rats with CLO treatment, and the increase in *UCG-008* and *Prevotella-7* observed with B-GOS treatment, were confirmed in Wisket rats.

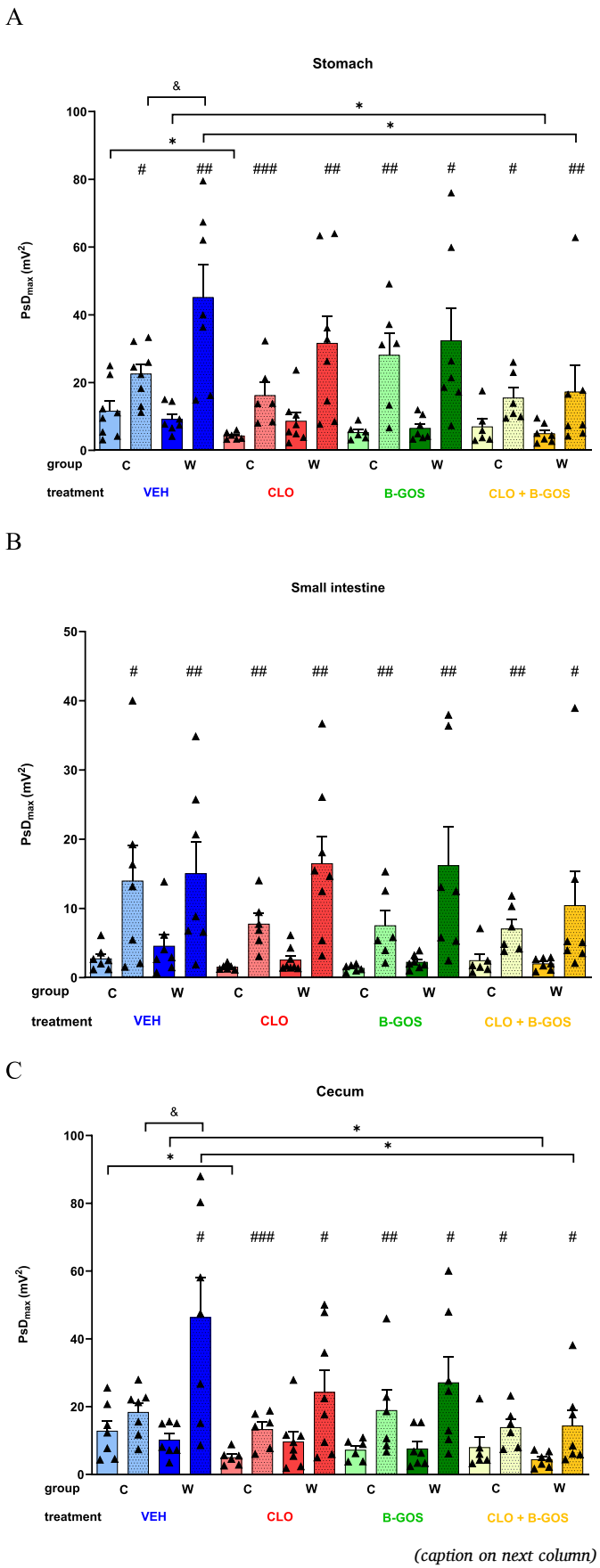
Overall, both CLO and B-GOS treatment could improve dysbiosis caused by the Wisket phenotype. However, many changes caused by CLO or B-GOS alone were not evident in animals treated with both, suggesting that these pharmacological interventions counteracted each other's effects, resulting in a weaker overall effect. This is consistent with behavioural results showing that combination treatment was less effective than monotreatments.

3.6. Correlation analysis

Since the most prominent differences between the treatment groups occurred during sessions 5 and 6 in posttreatment Ambitus test, these behavioural parameters were averaged and correlated with the microbiota profile. In the case of the SMEMG, the basal activity and the stress responses, with the most significant changes occurring in the stomach, were used.

Correlation analysis revealed a significant association between the microbiota profile and behavioural phenotype at different taxonomic levels (Supplementary Table S3, Supplementary Fig. S5). Several taxa associated with the schizophrenia model or pharmacological treatment were significantly correlated with behavioural parameters, some of these also significant after FDR correction was applied. The bacterial families most strongly associated with the investigated behavioural phenotype were *Bifidobacteriaceae, Erysipelatoclostridiaceae, Spirochaetaceae and Succinivibrionaceae*. As highlighted in Fig. 5, the microbiota-behaviour correlation network shows that the relative abundance of bacterial genera has the greatest influence on motivation index and working memory, evidenced by a node degree (i.e., number of significant correlations, not adjusting for multiple hypothesis testing) of 35 and 19, respectively. Considering q-values, motivation index exhibited a positive correlation with the relative abundance of *Bifidobacterium, Lactobacillus* and *Limosilactobacillus*. Both the motivation index and working memory were negatively correlated with *Erysipelotrichaceae UCG-003* and *Marvinbryantia*. These behavioural parameters also showed one additional negative correlation each with *Anaerobiospirillum* and the *[Ruminococcus] gnavus group*, respectively.

The microbiota-behaviour correlation network appears to be divided into two major modules, one organized around the cognition-related parameters featuring several strong associations, and the other centered on stress sensitivity and is rather uncertain. The only bacterial genera that seems to serve as a bridge between these two modules are *Anaerobiospirillum (Gammaproteobacteria)* and *UCG-005 (Clostridia)*. The stress sensitivity module is dominated by weak correlations (significance based on unadjusted p-values) with *Clostridia* and *Negativicutes* genera. However, the relevance of the stress sensitivity module and the link between the two modules is questionable, as none of the associations in these network structures were significant when adjusted for multiple hypothesis testing.



(caption on next column)

Fig. 3. Changes in the intensity of myoelectric signals (PsD_{max}) during immobilization stress (column with pattern) compared to basal activity (columns with no pattern) in the stomach (A), small intestine (B), and cecum (C) by group and by treatment. Data are presented as mean ± SEM, the small triangles represent individual data points. The symbols indicate significant differences compared to the corresponding basal activity (paired *t*-test, p values are indicated as follows: #p < 0.05, ##p < 0.01, and ###p < 0.005), between the groups (&, control vs Wisket counterparts, unpaired *t*-test, p < 0.05), and compared to the corresponding vehicle treatment (*, unpaired *t*-test, p < 0.05). Abbreviations: C – Control, W–Wisket, CLO – clozapine, B-GOS – Bimuno™ galactooligosaccharide, VEH – vehicle.

4. Discussion

Our study is the first to demonstrate that B-GOS prebiotic treatment is highly effective in improving behavioural deficits in a translationally relevant triple-hit schizophrenia rat model. By contrast, subchronic CLO treatment only produced moderate progress in cognitive performance in Wisket rats, which is consistent with most clinical and preclinical findings (Baldez et al., 2021; Gray et al., 2009; Levin and Christopher, 2006). Although, B-GOS and CLO had modest and diverse effects on normal microbiota composition, both treatments alleviated bacterial changes associated with the schizophrenia-like phenotype. Changes in distinct taxa showed a strong correlation with certain behavioural parameters, suggesting that the beneficial effects of B-GOS and CLO on schizophrenia-associated behavioural changes are likely due to their impact on gut microbiota composition. However, the combination of B-GOS and CLO appeared to be less effective in improving behavioural parameters and gut dysbiosis. This suggests that the beneficial mechanisms of the two treatments are different and may cancel each other out to some extent. Finally, in line with clinical findings in patients with schizophrenia, we also provided evidence of increased stress sensitivity in Wistar model rats (Taylor et al., 2019), which was alleviated by each pharmacological treatment.

Although, cognitive impairment is recognised as a core component of schizophrenia from which positive and negative symptom domains may arise, antipsychotic drugs have limited efficacy in treating it. While several authors argue that typical first-generation antipsychotics may worsen cognition (Kasper and Resinger, 2003), similar results have also been published regarding the atypical clozapine and risperidone (Baldez et al., 2021; Joshi et al., 2021; McGurk et al., 2005). Conversely, cognitive improvements have been reported, particularly with long-term CLO treatment, which is associated with enhanced glutamatergic neurotransmission (Buchanan et al., 1994; Galletly et al., 2005; Pereyra and Medina, 2021). Similar findings have been reported in rodent studies related to schizophrenia, with either no effect (Gray et al., 2009; Kamei et al., 2006), or further deterioration in working and recognition memory (Horvath et al., 2021; Levin and Christopher, 2006; Ortega-Alvaro et al., 2006). However, CLO has been shown to attenuate the cognitive deficits induced by phencyclidine and MK-801 treatment in rodents (Abdul-Monim et al., 2006). Antipsychotic drug use may interact with, and even alter, the gut microbiome, resulting in disruption to inflammatory cytokine signalling and altered neurotransmitter production (e.g. GABA, serotonin and BDNF). This can consequently lead to adverse metabolic effects and exacerbate cognitive deficits (Bora et al., 2017; Pyndt Jørgensen et al., 2015; Zeng et al., 2021; Zhang et al., 2017). In our Wisket model rats CLO treatment did not result in any prominent improvement in activity- or cognition-related parameters, probably due to their extremely low initial/original performance (floor effect) and altered microbiota composition. This includes reduced abundance of some genera of *Lachnospiraceae* and related butyrate production, along with still high relative abundance of *Erysipelotrichaceae-UCG-003*, considered as a pro-inflammatory microbe in humans and is associated with cognitive decline (Fusco et al., 2023; Kaakoush, 2015; Otto-Dobos et al., 2024; Ren et al., 2020). Nevertheless, further investigation is required to gain a detailed understanding of

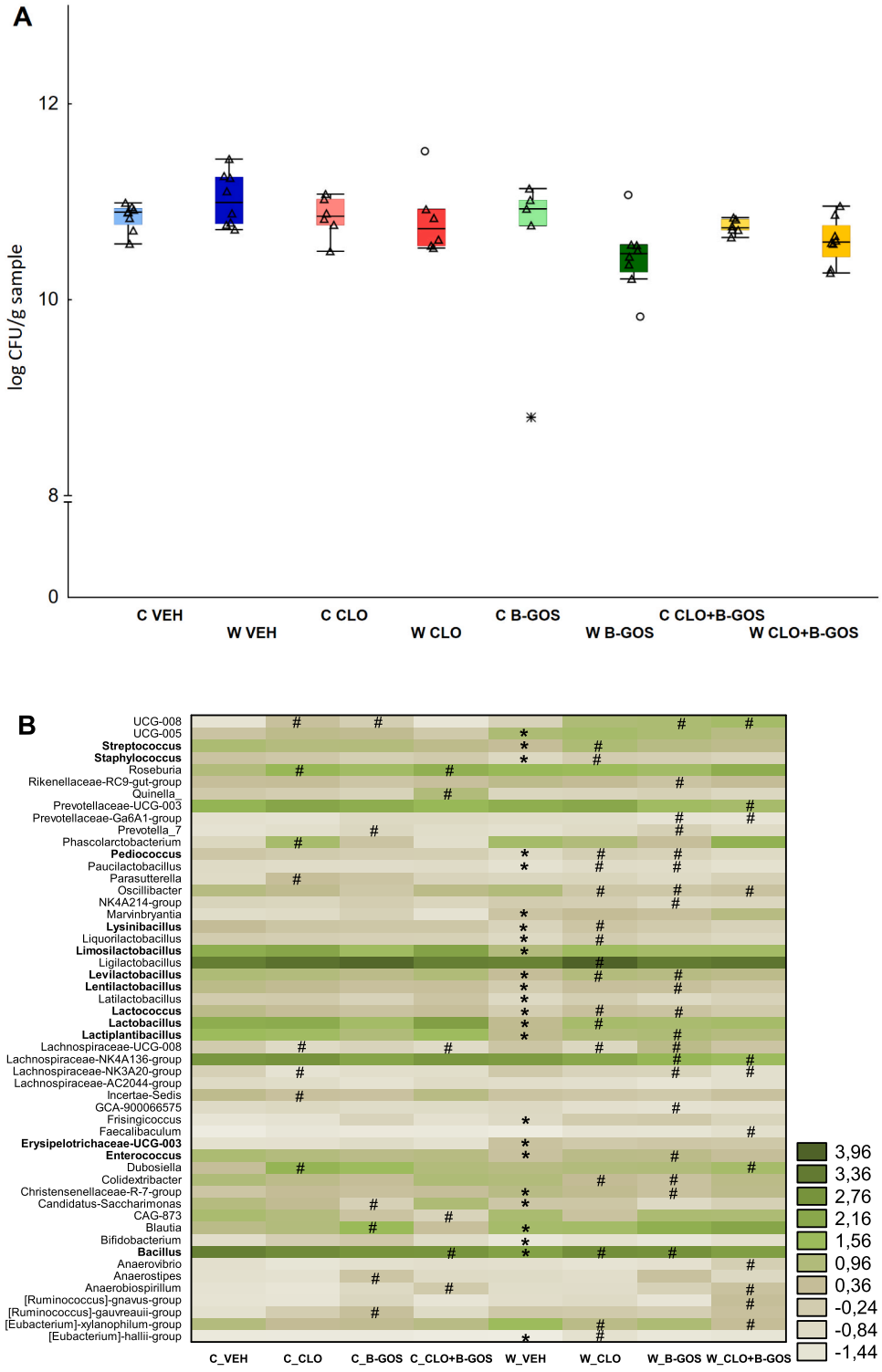


Fig. 4. A: Total bacterial amount by treatment groups determined by qRT-PCR. Box and whisker plots indicate the medians, first and third quartiles, and the minimum and maximum of non-outlier values, the triangles represent individual samples. **B**: Summary of the significant ($p < 0.05$) changes in bacterial genera by treatment groups using CLR values (Wilcoxon rank-sum test). Centered log ratio transformation was applied to reduce data skewness and compositionality bias in relative abundances. Taxa highlighted in bold indicate significant differential abundance after FDR correction ($q < 0.05$), too. The symbols indicate significant differences between the vehicle-treated groups (*), and by the pharmacological treatment compared to the corresponding vehicle-treated counterparts (#). Abbreviations: C – Control, W–Wisket, CLO – clozapine, B-GOS – Bimuno™ galactooligosaccharide, VEH – vehicle.

Table 2
Group differences at community level. Results of PERMANOVA analysis regarding the β-diversity at genus level (number of permutations: 10,000).

Sample size	Test statistic (pseudo-F)	p-value	Treatment groups
16	3.4021	0.0003	C_VEH vs W_VEH
14	2.1686	0.0124	C_CLO vs C_VEH
13	2.0534	0.0102	C_B-GOS vs C_VEH
14	1.6039	0.0206	C_CLO + B-GOS vs C_VEH
14	1.8696	0.0325	W_CLO vs W_VEH
16	1.9224	0.0254	W_B-GOS vs W_VEH
16	2.6327	0.0062	W_CLO + B-GOS vs W_VEH

Abbreviations: C – Control, W–Wisket, CLO – clozapine, B-GOS – Bimuno™ galactooligosaccharide, VEH – vehicle.

the underlying mechanisms, particularly through comprehensive metabolomic analyses.

Prebiotics may serve a dual therapeutic role in schizophrenia due to reducing low-grade inflammation by inhibiting pro-inflammatory signals, which is harmful to both cognitive function and metabolic processes (Kao et al., 2018b, 2019; Mallappa et al., 2012). For example, the adjunctive treatment with B-GOS has been shown to alter TNF-α and IL-1 levels in patients with schizophrenia, which has also been associated with the elevated levels of circulating acetate (Kao et al., 2018a). It has also been found to significantly improve cognitive flexibility in rats alleviating NMDA hypofunction and elevating hippocampal BDNF level (Gronier et al., 2018; Savignac et al., 2013). Prebiotics have also been shown to restore cognition in rats with obesity-related insulin resistance and chronic ketamine-induced schizophrenia due to improved hippocampal plasticity, enhanced brain mitochondrial function, reduced microglial activation, inhibition of the kynurenic acid metabolic pathway and decreased neuroinflammation (Chunchai et al., 2018; Xu et al., 2025). A recent study using a single-hit genetic mouse model of schizophrenia reported that a prebiotic combination treatment had a beneficial effect, rescuing gut microbiome changes related to the knockout model and improving cognition (Gubert et al., 2025). In our present study, B-GOS alone resulted in a more pronounced improvement than the CLO + B-GOS combination treatment. Although the explanation requires further clarification, theoretically, it may be linked to the complex interactions between gut bacteria and combined drug treatments. The different microbiome compositions in the various groups may influence drug metabolism. Alternatively, the two drugs may alter the microbiome in distinct ways, thereby impacting drug responses (Trepka et al., 2025). This is partly supported by the fact that B-GOS and

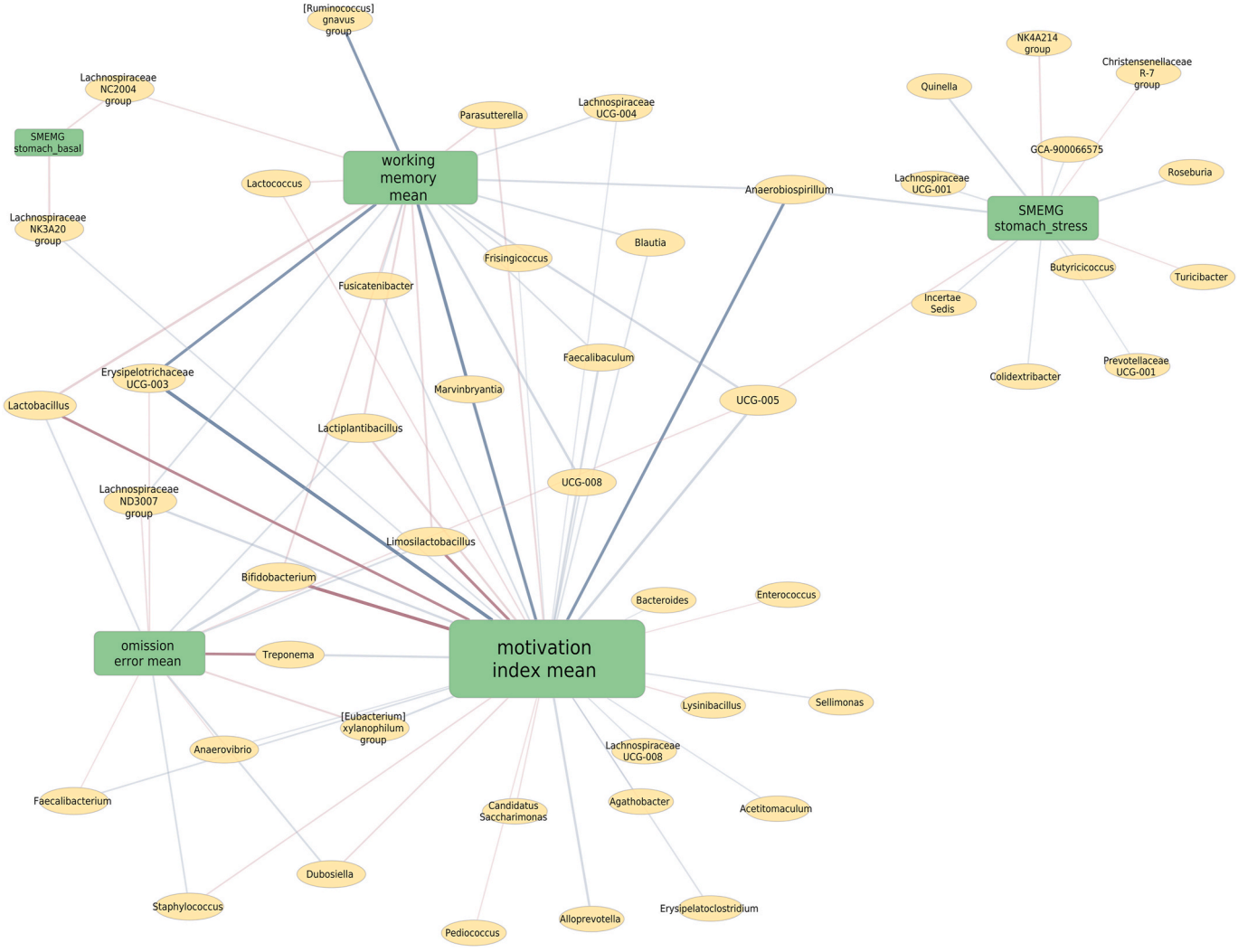


Fig. 5. Microbiota-behaviour correlation network. In this bipartite graph nodes represent behavioural parameters (green) and microbiota genera (yellow). Edges connect nodes that showed a significant positive (red) or negative (blue) correlation before ($p < 0.05$; $q \geq 0.05$; pale color) and after ($q < 0.05$; bright color) adjustment for multiple hypothesis testing. Thickness of the edges is proportional to the absolute value of the Spearman's rank correlation coefficient (Rho-value), while node size is proportional to the node degree (i.e., the number of incident edges). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

CLO treatments affected the relative abundance of different bacteria, and that the combination treatment had only a minimal effect on the observed microbiota differences in the Wisket phenotype. Additionally, previous literature has indicated that prebiotic treatment increases hippocampal BDNF levels (Romo-Araiza et al., 2026), whereas certain antipsychotics decrease them in association with exacerbated cognitive impairment and metabolic syndrome (Zhang et al., 2017). Furthermore, it might be associated with an increased abundance of several genera of *Lactobacillaceae* and *Lachnospiraceae*, including *Marvinbryantia* and the *[Ruminococcus]-gnavus-group*.These genera are responsible for producing lactic acid, SCFA and serotonine, assuming their potential positive effects on cognition and mental disorders (Fusco et al., 2023; Hsieh et al., 2020; Son et al., 2025). It is also negatively associated with the differential abundance of *Erysipelotrichaceae-UCG-003,* which presumably indicates improved cognition (Otto-Dobos et al., 2024).

Early-life stress, mimicked in our rat models by the hit of isolation rearing, can cause lasting changes in HPA axis regulation. This increases susceptibility to the disorder and exacerbates cognitive symptoms (Mikulska et al., 2021). Dysregulation of the HPA axis may involve altered glucocorticoid levels and sensitivity of the glucocorticoid receptor (Nikolić et al., 2024). It is also associated with inflammation and oxidative stress, which worsens neurodegeneration in schizophrenia. For example, it is linked to reduced hippocampal volume, contributing to cognitive impairments (Mondelli et al., 2010; Saad et al., 2025). In the present study we measured the myoelectric activity along the gastrointestinal tract, which correlates with smooth muscle contraction and stress-related autonomic parameters. that shows correlation with smooth muscle contraction, stress-related autonomic parameters and plasma cortisol levels. (Pribék et al., 2021; Szűcs et al., 2018). In consistent with clinical and preclinical observations, our SMEMG results indicated an elevated stress response in the Wisket model of schizophrenia (Mikulska et al., 2021; Zimmerman et al., 2013), which was associated with the differential abundance of several bacterial taxa. These included *Clostridia* (negatively correlated with *Lachnospiraceae* and positively with *Oscillospiraceae* and *Christensenellaceae*), *Negativicutes, Erysipelotrichia* and *Proteobacteria* (Łopucki et al., 2025; Ying et al., 2025). However, it should be noted that there are large individual differences in stress response within the Wisket group, which also correlate well with clinical observations of elevated baseline cortisol and challenge-induced HPA activity in some, but not all, patients with schizophrenia compared to controls (Bradley and Dinan, 2010; Walker et al., 2008).

Both preclinical and clinical evidence suggests that atypical antipsychotics have the potential to protect against stress responses by reducing glucocorticoid receptor activation or cortisol levels (Nikolić et al., 2024). CLO-induced hypomotility throughout the whole gastrointestinal tract is a well-known phenomenon. Preclinical data have also demonstrated that CLO inhibits the contraction of smooth muscles in the rabbit colon and urinary bladder (Every-Palmer et al., 2017; Obara et al., 2021). Similarly, our present study confirmed this by demonstrating significantly reduced basal myoelectric activity in CLO-treated control and CLO + B-GOS co-treated Wisket animals. These results are not solely due to anticholinergic and/or antiserotonergic features; studies have suggested that the dopaminergic and histaminergic systems may also be implicated in CLO's adverse impact on intestinal motility (Xu et al., 2021). Additionally, our present study found that some bacterial taxa were also associated with altered basal SMEMG activity, including reduced abundance of some *Lachnospiraceae* in CLO-treated animals.

Resident gut microbiota affects the characteristics of the intestinal myoelectric activity and consequently the gut motility response (Husebye et al., 2001). The gut microbiome produces neurotransmitters, including acetylcholine, catecholamines, gamma-aminobutyric acid, histamine and serotonin. It influences behaviour and is essential for regulating gastrointestinal peristalsis, as well as being vital for absorption, transport and clearance (Hoffman et al., 2020). An increasing amount of preclinical and clinical evidence supports the idea that enteric bacteria and the gut-brain axis play a significant role in developing resilience or susceptibility to stress and in regulating the HPA axis, associated with higher incidence of neuropsychiatric disorders (Mikulska et al., 2021; Sah et al., 2024). A preclinical study also reported elevated corticosteroid levels in germ-free mice subjected to restraint stress, alongside decreased BDNF and NMDA receptor expression in the hippocampus and cortex, compared to specific pathogen-free mice (Sudo et al., 2004). Furthermore, dietary supplementation with prebiotics, such as B-GOS, fructooligosaccharides, polydextrose and oligosaccharide esters from *Polygalae radix*, has been shown to mitigate stress-related behaviour, HPA-axis functioning and gut microbiota in rodents (Burokas et al., 2017; Chen et al., 2023; O'Mahony et al., 2020). The group difference in stress response disappeared with each pharmacological intervention, with the most prominent effect of CLO + B-GOS combination treatment. In the context of the literature, this might be related to the reduced abundance of *Lachnospiraceae-UCG-008* and the elevated abundance of *Roseburia*, which in the context of the literature might be related to reduced abundance of *Lachnospiraceae-UCG-008* and elevated abundance of *Roseburia* (Zhang et al., 2019).

We must acknowledge some limitations to this study. First, the relatively small sample size meant that the animals could not be subgrouped based on their individual stress sensitivity; thus, using the mean of highly variable data within a group could mask group differences and increase the risk of false negatives. Second, although symptom severity in schizophrenia, stress sensitivity and microbiota composition all exhibit sex-dependent differences, the study only involved male rats. Based on our previous observations, we found that male rats showed higher sensitivity to the triple hit. Furthermore, in line with the principle of reduction, we were able to minimise the number of animals. Third, keeping the animals in pairs and allowing them to consume the prebiotic freely from a shared drinking bottle influenced precise dosing and made it difficult to determine the exact cause of the distinct behavioural and microbial outcomes between the groups. Although oral gavage is a more precise method, voluntary consumption is a less stressful alternative for pharmacological studies. It was particularly important to minimise stress as a confounding factor, given that the stress response was one of our endpoints of interest. However, our findings in vehicle-treated Wisket model rats are highly correlated with our previous findings when analysing untreated Wisket model rats. Fourth, the food-rewarded protocol can acutely alter the composition of the gut microbiota, so faecal sampling would probably have been better performed before behavioural testing. However, the weight of the reward consumed compared to the normal laboratory chow consumed was negligible, and the treatment efficacy was primarily compared to vehicle-treated counterparts, showing no significant difference in this parameter.

In conclusion, this proof-of-concept study supports the idea first that B-GOS prebiotic treatment might be highly effective in improving cognition-related behavioural deficits and hypersensitivity to stress in a translationally relevant triple-hit schizophrenia rat model, in association with fecal microbiota composition. Furthermore, its beneficial effects in healthy control animals were also verified. The study indicates the microbiome-gut-brain axis as a potential drug target in neuropsychiatric disorders. However, as these results are derived from a preclinical model, they should be treated with caution, and substantial additional research is required to elucidate the underlying mechanisms and enable translation through subsequent preclinical and clinical studies.

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CRediT authorship contribution statement

Szonja B. Plesz: Conceptualization, Investigation, Visualization, Writing – original draft. **Kálmán F. Szűcs:** Investigation, Methodology, Visualization. **Bence Ágg:** Formal analysis, Writing – original draft. **Nóra Makra:** Investigation. **Balázs Ligeti:** Formal analysis. **Zsuzsanna O. Demeter:** Investigation, Visualization. **Leatitia G. Adlan:** Investigation. **Péter Liszli:** Methodology. **Dóra Szabó:** Writing – review & editing. **Péter Ferdinandy:** Writing – review & editing. **Zoltán S. Zádori:** Supervision, Writing – review & editing. **Róbert Gáspár:** Conceptualization. **Gyongyi Horvath:** Writing – review & editing. **Gabriella Kekesi:** Conceptualization, Methodology, Visualization, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The graphical abstract was created with BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2026.110946.

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