1   **Identification of gut microbiome markers for schizophrenia delineates a**

2   **potential role of *Streptococcus***

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46   **Abstract**

47   Emerging evidence has linked the gut microbiota to schizophrenia. However, the

48   functional changes in the gut microbiota and the biological role of individual bacterial

49   species in schizophrenia have not been explored systematically. Here, we

50   characterized the gut microbiota in schizophrenia using shotgun metagenomic

51   sequencing of feces from a discovery cohort of 90 drug-free patients and 81 controls,

52   as well as a validation cohort of 45 patients taking antipsychotics and 45 controls. We

53   screened 83 schizophrenia-associated bacterial species and constructed a classifier

54   comprising 26 microbial biomarkers that distinguished patients from controls with a

55   0.896 area under the receiver operating characteristics curve (AUC) in the discovery

56   cohort and 0.765 AUC in the validation cohort. Our analysis of fecal metagenomes

57   revealed that schizophrenia-associated gut–brain modules included short-chain fatty

58   acids   synthesis,   tryptophan   metabolism,   and   synthesis/degradation   of

59   neurotransmitters including glutamate, γ-aminobutyric acid, and nitric oxide. The

60   schizophrenia-enriched gut bacterial species include several oral cavity-resident

61   microbes,   such   as   *Streptococcus   vestibularis*.   We   transplanted   *Streptococcus*

62   *vestibularis* into the gut of the mice with antibiotic-induced microbiota depletion to

63   explore its functional role. We observed that this microbe transiently inhabited the

64   mouse gut and this was followed by hyperactivity and deficit in social behaviors,

65   accompanied with altered neurotransmitter levels in peripheral tissues. In conclusion,

66   our study identified 26 schizophrenia-associated bacterial species representing

67   potential microbial targets for future treatment, as well as gut–brain modules, some of

68   which may give rise to new microbial metabolites involved in the development of

69   schizophrenia.

Introduction

Schizophrenia is a severe psychiatric disorder associated with hallucinations, delusions, and thought disorders perturbing perception and social interaction[1]. The etiology of schizophrenia is not elucidated, but assumed to be multifactorial involving genetic and environmental factors. Abnormalities of neurotransmitter systems have been extensively studied especially focusing on aberration of signaling involving dopamine, glutamate, and γ-aminobutyric acid (GABA)[2-4]. Increasing evidence indicates that schizophrenia may be a systemic disorder with neuropsychiatric conditions in addition to psychosis[5]. Furthermore, the importance of inflammation[6] and the involvement of the gastrointestinal system[7] in schizophrenia have received attention.

The gut microbiota is reported to play an important role in neurogenerative processes, and perturbation of the microbiota and microbial products have been demonstrated to affect behavior[8-10]. Changes in the gut microbiota have been associated with neurological[11] and neurodevelopmental disorders[12,13], including schizophrenia[14]. It was recently reported that fecal transfer of the gut microbiota from patients with schizophrenia induces schizophrenia-associated behaviors in germ-free recipient mice accompanied with altered levels of glutamate, glutamine, and GABA in the hippocampus[13]. However, the identity and functionality of the specific bacteria responsible for mediating changes in the behavior of recipient mice are unknown[15,16]. Thus, the composition and functional capacity of the gut microbiota in relation to schizophrenia need to be systematically examined. Taxa and functional profiling of the microbiota composition is a premise for functional understanding of the gut microbiota[17]. Previous studies were based on 16S rRNA gene amplicon sequencing of relatively small cohorts[14,18-20] and hence failed to provide detailed functional profiling of the gut microbiota in schizophrenia. Metagenomic shotgun sequencing

96   combined with bioinformatics tools enables better characterization of the

97   microbiota[21], including a more accurate prediction of biological features of the

98   microbes and their impacts on host physiology[22].

99   Here, we carried out a metagenome-wide association study (MWAS) using

100   171 samples (90 cases and 81 controls) and validated the results with an additional 90

101   samples (45 cases and 45 controls). The functional changes within the schizophrenia

102   gut microbiota were determined using pathway/module analysis based on the Kyoto

103   Encyclopedia of Genes and Genomes (KEGG) and a recently developed gut–brain

104   module (GBM) analysis of fecal metagenomes[23]. The functional roles of one

105   particular schizophrenia-enriched gut bacterial species, *Streptococcus vestibularis*,

106   were explored by transplanting this bacterium into the gut of the mice with antibiotic-

107   induced microbiota depletion and observing its effects on animal behavior and brain

108   neurochemicals.

109

110   **Results**

111   **The gut microbiota differs between schizophrenic patients and healthy controls**

112   We carried out shotgun sequencing on fecal samples from 90 drug-free patients and

113   81 healthy controls (for demographic and clinical characteristics see Supplementary

114   Table 1-3) and obtained an average of over 11 gigabases (Gb) sequence data per

115   sample and mapped the high-quality reads onto a comprehensive reference gene

116   catalogue of 11.4 million genes[24] (Supplementary Table 4).

117   The gut microbiota in schizophrenic patients showed greater α diversity at the genus

118   level ($P = 0.027$, Wilcoxon rank-sum test), higher β diversity at the genus level ($P <$

119   0.001, Wilcoxon rank-sum test) and microbial gene level ($P < 0.001$, Wilcoxon rank-

120   sum test) and comprised more genes compared with healthy controls (Supplementary

5

121 Fig. 1). Out of a total of 360 metagenomic operational taxonomic units (mOTUs)[25],

122 83 mOTUs showed significant differences in relative abundance between patients and

123 controls ($P < 0.05$ and false discovery rate (FDR) = 0.136, Wilcoxon rank sum test

124 and Storey's FDR method; Supplementary Table 5a). After adjusting for BMI, age,

125 sex, and diet, these 83 mOTUs were still significant (Supplementary Table 5a). The

126 gut microbiota in schizophrenic patients harbored many facultative anaerobes such as

127 *Lactobacillus fermentum, Enterococcus faecium, Alkaliphilus oremlandii*, and

128 *Cronobacter sakazakii/turicensis*, which are rare in a healthy gut. Additionally,

129 bacteria that are often present in the oral cavity, such as *Veillonella atypica*,

130 *Veillonella dispar*, *Bifidobacterium dentium*, *Dialister invisus*, *Lactobacillus oris,* and

131 *Streptococcus salivarius* were more abundant in patients with schizophrenia than in

132 healthy controls, indicating a close association between the oral and the gut

133 microbiota in schizophrenia.

134 We then constructed a mOTU network to depict the co-occurrence correlation

135 between the schizophrenia-associated gut bacteria (Fig. 1). Schizophrenia-enriched

136 mOTUs (Wilcoxon rank-sum test) were more interconnected than control-enriched

137 mOTUs (Spearman's correlation coefficient < -0.3 or >0.3, $P < 0.05$). The mOTU

138 species from the genera *Streptococcus* and *Veillonella* showed positive cross-

139 correlations. Moreover, the majority of the species in these two clusters of correlated

140 mOTUs originated from the oral cavity, suggesting that orally resident bacteria in a

141 synergistic manner may colonize the gut in schizophrenic patients (Fig. 1 and

142 Supplementary Table 5a).

143 Functional modules and pathways enriched in the gut microbiota of patients relative

144 to controls were analyzed using the KEGG database (Supplementary Table 6). The

145 relative enrichment of 579 KEGG modules and 323 KEGG pathways varied

6

146 significantly between the two groups. Schizophrenia-depleted microbial functional

147 modules included pectin degradation, lipopolysaccharide biosynthesis, autoinducer-2

148 (AI-2) transport system, glutamate/aspartate transport system, beta-carotene

149 biosynthesis, whereas schizophrenia-enriched functional modules included

150 methanogenesis, the gamma-aminobutyrate (GABA) shunt, and transport system of

151 manganese, zinc, and iron (Supplementary Table 6).

152 **Neuroactive potential of schizophrenia-related bacterial species**

153 We next compared the altered microbial neuroactive potential of the gut microbiota

154 of schizophrenic patients with the controls at the species level using the method

155 reported by Valles-Colomer et al.[23]. We mapped the metagenomic data of the 171

156 samples to a genome database including the 42 microbial species that were detected

157 based on the 83 schizophrenia-associated mOTUs using PanPhlAn[26] and calculated

158 the prevalence of species-level microbes. We then determined whether the abundance

159 of 56 previously reported gut-brain modules (GBMs)[23], present in each microbial

160 species, varied significantly between schizophrenic patients and controls. The GBM

161 set of each microbial species was obtained by cross-checking GBM-related genes and

162 the species gene repertoires (Supplementary Table 7a). The frequency of the

163 occurrence of each GBM within each species was compared between patients and

164 controls using a Chi-squared test (Supplementary Table 7b). Schizophrenia-associated

165 GBMs included short-chain fatty acid synthesis (acetate, propionate, butyrate, and

166 isovaleric acid), tryptophan metabolism, and the synthesis of several

167 neurotransmitters, such as glutamate, GABA, and nitric oxide (Fig. 2).

168 We chose to validate the presence of the GBM associated with tryptophan

169 metabolism in schizophrenia, as tryptophan metabolism also is modulated by the gut

170 microbiota and implicated in schizophrenia pathogenesis[27,28]. Hence, serum

7

171 tryptophan metabolites were measured in patients and controls and correlated with the

172 presence of tryptophan modules in the gut microbiota. In agreement with the higher

173 abundance of tryptophan metabolisms related GBMs, we observed lower serum

174 tryptophan levels and higher kynurenic acid (KYNA) levels in schizophrenic

175 patients(Supplementary Fig. 2a, c). Moreover, serum tryptophan levels were

176 negatively correlated with the abundances of 38 bacterial species enriched in

177 schizophrenic patients and positively correlated with six bacterial species enriched in

178 controls (Supplementary Fig. 2d). Similarly, serum KYNA levels were positively

179 correlated with 10 schizophrenia-enriched bacterial species and negatively correlated

180 with 3 control-enriched bacterial species (Supplementary Fig. 2d). Thus, an altered

181 gut microbiota may be associated with changes in serum levels of tryptophan and

182 KYNA in schizophrenia.

183 **Microbial species-based biomarkers for schizophrenia**

184 To identify novel gut bacterial biomarkers and evaluate their diagnostic values for

185 schizophrenia, we first constructed a set of random forest disease classifiers based on

186 gut mOTUs. We performed a five-fold cross-validation procedure ten times on 90

187 patients and 81 controls. Twenty six gut mOTUs reached the lowest classifier error in

188 the random forest cross validation, and the ROC AUC score of the model was 0.896

189 (Fig 3a, b). This microbial based classifier was not significantly influenced by age,

190 gender, BMI, and diet style (Supplementary Table 8). This discriminatory model was

191 validated on an additional validation cohort consisting 45 patients taking

192 antipsychotics and 45 controls (Supplementary Table 9). The model still distinguished

193 patients from controls with an ROC AUC score of 0.765. Among the 26 microbial

194 biomarkers, 11 bacterial species with taxonomic identity were significantly enriched

195 in schizophrenia, namely *Akkermansia muciniphila*, *Bacteroides plebeius*, *Veillonella*

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196 *parvula, Clostridium symbiosum, Eubacterium siraeum, Cronobacter*

197 *sakazakii/turicensis, Streptococcus vestibularis, Alkaliphilus oremlandii,*

198 *Enterococcus faecium, Bifidobacterium longum*, and *Bifidobacterium adolescentis.*

199 Some of these microbial biomarkers were significantly associated with symptom

200 severity, cognitive performance, and diagnosis (Fig 3c). We next performed

201 metagenomic analysis on the fecal samples from 38 of the 90 patients after 3-months

202 of treatment (27 with risperidone and 11 with other antipsychotics, shown in

203 Supplementary Table 1). The psychotic symptoms and cognitive impairment

204 improved greatly along with treatment (Supplementary Fig. 3). However, only

205 approximately half of the microbial biomarkers returned to the levels in controls after

206 treatment (Fig. 3d). As the sample size of the follow-up patients was smaller, the

207 statistical significance threshold was increased from 0.05 to 0.1. Of the 26 identified

208 microbial biomarkers, 20 biomarkers remained significantly changed between 81

209 controls and 38 baseline patients ($P < 0.1$, FDR = 0.44, Benjamini and Hochberg

210 method, Fig. 3d). After 3-months of treatment, the abundances of 12 of these 26

211 biomarkers remained significantly changed compared with 81 controls ($P < 0.1$, FDR

212 = 0.33, Benjamini and Hochberg method, Supplementary Table 10). Pair-wise

213 comparison of all gut mOTUs for the treatment effect in the follow-up patients

214 revealed 48 differentially abundant bacterial species ($P < 0.05$ and FDR = 0.420,

215 Paired Wilcoxon rank sum test; Benjamini and Hochberg method, Supplementary

216 Table 11). However, only 5 of the 48 differentially abundant species were included in

217 the 26 microbial biomarkers used for diagnosis. This result suggests that antipsychotic

218 treatment influences the gut microbiota, but does not completely restore the altered

219 microbiota associated with schizophrenia.

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221 **_Streptococcus vestibularis induced schizophrenia-like behaviors in mice_**

222 _Streptococcus_ (_S._) _vestibularis_ was identified as a diagnostic marker associated with

223 serum GABA, tryptophan, KYNA, and the Brief Assessment of Cognition in

224 Schizophrenia (BACS) scores in MATRICS Consensus Cognitive Battery (MCCB) test

225 (Fig. 3 and Supplementary Fig. 2). Moreover, _S. vestibularis_, present in the gut of a

226 number of schizophrenic patients, was predicted to have GBMs related to glutamate

227 synthesis, GABA degradation, and Isovaleric acid synthesis I (Fig. 2). As some

228 pathogenic species of _Streptococcus_ are known to enter the brain[29], and have been

229 implicated in pediatric acute-onset neuropsychiatric syndrome[30,31], we asked if _S._

230 _vestibularis_ might play a role in the pathophysiology of schizophrenia. Hence, we

231 transplanted _S. vestibularis_ ATCC 49124, using oral gavage and drinking water, into

232 C57BL/6 mice after antibiotics-based microbiota depletion (Supplementary Fig. 4).

233 Another strain of streptococcus, _S. thermophilus_ ST12, which is widely present in the

234 human gut, was used as a bacterial control. Behavioral tests were performed to

235 evaluate the effect of _S. vestibularis_ transplantation (Fig. 4a). Quantitative polymerase

236 chain reaction (q-PCR) used to quantify the 16S rRNA gene of _S. vestibularis_ and _S._

237 _thermophilus,_ revealed that their concentration increased by 4,164- and 6,183-fold

238 immediately after transplantation and remained at a 31.2- and 58.1-fold increase after

239 the behavioral tests as compared to the control mice (Supplementary Fig. 5).

240 Compared to control mice gavaged with buffer or with S. thermophilus, the S.

241 vestibularis-treated mice had increased their total distance traveled and times of

242 rearing during a 30-minute open-field test (Fig. 4b in this figure, it is better to put S.

243 thermophilus next to control since S. thermophilus is also a control, d). They

244 continued their hyperlocomotion after the 10-minute habituation period and showed

245 no obvious decline in locomotion activity after a period of 30-minutes (Fig. 4c). In the

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246   three-chamber social test, the S. vestibularis mice displayed obvious deficits in

247   sociability and social novelty, as they were much less sociable and avoided social

248   novelty (Fig. 4e-g). However, in Barnes maze, elevated plus maze, and tail suspension

249   test, the mice transplanted with *S. vestibularis* displayed spatial memory function,

250   depressive state, and anxiety levels similar to either saline or *S. thermophilus*-treated

251   mice (Supplementary Fig. 6). There were no significant changes in body weight,

252   systemic pro-inflammatory cytokines, and endotoxin, and HPA axis hormones among

253   the S. vestibularis-treated, S. thermophilus-treated, and control mice (Supplementary

254   Fig. 7 a-i).

255   We then compared the transcriptome and neurotransmitter levels between *S.*

256   *vestibularis*-treated and saline-treated mice in their peripheral tissues and brain. *S.*

257   *vestibularis* treated mice had significantly lower levels of dopamine in serum,

258   intestinal contents, and colonic tissue, as well as decreased GABA levels in the

259   intestinal contents immediately after the transplantation, but these effects disappeared

260   after 10 days of post-transplantation (Supplementary Fig. 8b, e, h, and f). Intestinal

261   contents of S. vestibularis-treated mice showed increased levels of 5-HT throughout

262   the behavioral tests (Supplementary Fig. 8d). S. vestibularis transplantation did not

263   induce obvious inflammatory cell infiltration (Supplementary Fig. 6j) but induced

264   changes in the expression of numerous immune/inflammation-related genes in the

265   intestine when compared with saline gavage (Supplementary Table 12 a-b). By gene

266   enrichment anslysis, we found these genes were enriched in cytokine-cytokine

267   receptor interaction, chemokine signaling pathway, leukocyte trans-endothelial

268   migration, complement and coagulation cascades, antigen processing and

269   presentation, intestinal immune network for IgA production, and inflammatory bowel

270   disease (Supplementary Fig. 9a-b). These results suggested that *S. vestibularis* may

271   influence gut immune homeostasis. In the brain, the levels of neurotransmitters were

272   not affected by transplantation with *S.* vestibularis and only tryptophan decreased in

273   the prefrontal cortex of S. vestibularis-treated mice (Supplementary Table 13).

274   However there are 354, 540, and 470 significantly differentially expressed gene in the

275   PFC, striatum, and hippocampus between *S. vestibularis*-treated and saline treated

276   mice (Supplementary Table 12 c-d). The pathways influenced by these differentially

277   expressed genes included defense responses and immune-regulating pathways like in

278   the gut, as well as peroxisome proliferator-activated receptor signaling pathway,

279   steroid biosynthesis, tyrosine, and tryptophan metabolism (Supplementary Fig. 9c-e).

280   **Discussion**

281   We here used a combination of MWAS and a functional-based approach to

282   systematically screen for schizophrenia-associated gut microbes and identified a

283   number of schizophrenia-associated GBMs, expanding our insight into functional

284   changes characterizing the gut microbiota of schizophrenic patients. Consistent with

285   the MWAS results and the inferred microbial functions, the schizophrenia-associated

286   bacterium *Streptococcus vestibularis* was shown in mice to have a functional

287   neuroactive potential[23] associated with changes in animal behaviors.

288   Poor reproducibility is common in microbiome research[32]. In the present study, a

289   diagnostic model of 26 microbial biomarkers obtained using a discovery cohort,

290   which included drug-free patients, was well validated in a testing cohort, which

291   included patients taking antipsychotics. We chose to investigate drug-free patients in

292   the discovery set in order to eliminate the possible effects of antipsychotics on the gut

293   microbiota and identify gut bacteria possibly involved in the development of

294   schizophrenia. The validation of the initial findings in patients taking antipsychotics

295   demonstrated that these microbial markers are, to a certain extent, independent of

296 antipsychotics. Follow-up analysis also revealed that 20 of the 26 identified microbial

297 biomarkers in the diagnostic model remained the same over a treatment duration of

298 three months. Therefore, most microbial biomarkers for schizophrenia seem stable

299 and are not sensitive to current antipsychotics.

300 Analysis of the bacterial V3-V4 region of 16S rRNA regions has a limited resolution

301 in terms of identification of bacterial species[17,25,33]. Current 16S rRNA gene amplicon

302 sequencing generally capture reliable taxonomic classification at the genus level[33].

303 However, several recent analyses indicate that many taxonomic associations might be

304 presented only at levels subordinate to species[17,34,35]. Accordingly, most of the

305 schizophrenia-associated microbial species revealed by the MWAS results were not

306 identified in previous studies by using 16S rRNA gene sequencing[14,18,36]. There are

307 more overlaps of findings at genus level between our findings and the previous

308 studies (Supplementary Table 5b), including 6 in the study of Zheng et al.

309 (*Acidaminococcus, Akkermansia, Alistipes, Citrobacter, Dialister, Veillonella*)[14], 1 in

310 the study of Schwarz et al. (*Lactobacillus*)[36], 1 in the study of Shen et al.

311 (*Methanobrevibacter*)[18]. Surprisingly, gut microbiota diversities based on genus level

312 taxonomy and annotated genes were higher in schizophrenic patients compared to

313 controls. In accordance with our data, both α diversity and β diversity showed an

314 increase in the blood microbiota of schizophrenic patients[37]. The microbes in blood

315 are thought to originate from the gut as well as from the oral cavities[38,39]. Moreover,

316 the increased diversity of the blood microbiota may be due to the nonspecific overall

317 increased microbial burden in schizophrenia[37], which is supported by our observed

318 increased microbial gene number in patients' gut. The considerable heterogeneity of

319 the etiology and clinical manifestation of schizophrenia[40,41] may be implicated in such

320 an increase in the microbiota diversity. Another notable feature of the gut microbiota

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321   in schizophrenia is the significant enrichment of oral cavity resident bacteria.

322   Increased bacterial translocation due to a leaky gut and innate immune imbalance are

323   both presented in patients with schizophrenia[42,43]. Furthermore, gastrointestinal

324   inflammation due to a dysfunctional immune response to pathogen infection and food

325   antigens is also prevalent in schizophrenia[44,45]. These intestinal pathological

326   conditions may disrupt the mucosal barrier and decrease immune surveillance towards

327   foreign microbes[7], increasing the possibility of observing oral bacteria in the gut.

328   The composition of human gut microbiota is linked to schizophrenia[14,18,36], but

329   knowledge of individual microbial species is needed to decipher their biological

330   role[46]. We still do not completely understand the functions of most of the

331   schizophrenia-associated microbes identified here in the gut or their biological roles

332   in schizophrenia. Intriguingly, some schizophrenia-enriched bacterial species in the

333   present study are also over-presented in subjects with metabolic disorders and

334   atherosclerotic cardiovascular diseases[47-50]. Schizophrenic patients are more likely to

335   develop obesity, hyperglycemia/diabetes, hypertension, and cardiovascular disease.

336   Additionally, some of these risks are independent of the effects of antipsychotic

337   administration and healthy lifestyle choices[51-54]. Several prenatal and early-life risk

338   factors are shared by schizophrenia, metabolic disorders, and cardiovascular diseases

339   such as prenatal famine, postnatal growth restriction, the quality of fetal growth, and

340   low birth weight[55-61]. Gut microbes enriched in both schizophrenia and metabolic

341   disorders/cardiovascular disease may account for the increased risk of these

342   comorbidities in schizophrenia. Moreover, new evidence also indicates that metabolic

343   disorders in schizophrenia are not only comorbidities, but also affect pathogenesis,

344   such as the manifestation of negative symptoms[62], cognitive function[63], and brain

345   white matter disruption[64]. Treating metabolic disorders via physical activity and

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346   psychosocial and dietary interventions, is also an effective approach to improve the

347   symptoms of schizophrenia[63]. Therefore, manipulation of gut microbes may have

348   double therapeutic potential for both metabolic disorders and schizophrenia.

349   Identifying the mediators of gut–brain microbiota communication affected by

350   specific microbial metabolites is a key step to elucidate the mechanism behind the

351   regulation of the host's behaviors and psychology by gut microbial species. A recently

352   published database of manually curated GBMs provides a powerful tool for functional

353   interpretation of metagenomes in a microbiota–gut–brain context[23]. Using this

354   database, we identified 27 schizophrenia-associated GBMs, which give clues about

355   how the gut microbiota might modulate the pathophysiology of schizophrenia. Among

356   these GBMs, a few well-known molecular entities associated with schizophrenia were

357   covered, such as several kinds of neurotransmitters[2-4] and tryptophan metabolites[65,66].

358   Furthermore, some microbes presenting these GBMs were significantly associated

359   with the serum levels of several neurotransmitters and tryptophan metabolites.

360   Parallel to the present study, our previous animal study demonstrated that

361   transplantation of fecal microbiota from drug-free patients with schizophrenia into

362   specific pathogen-free mice could cause schizophrenia-like behavioral abnormalities

363   and dysregulated kynurenine metabolism[67]. The consistent findings of altered

364   tryptophan-kynurenine metabolism revealed by human serum metabolite analysis,

365   microbiota-based GBM prediction, and mouse studies suggest that this pathway is an

366   important link between schizophrenia and gut microbiota dysbiosis. Of note,

367   transplantation of one bacteria, *S. vestibularis ATCC 49124*, possessing a GBM

368   involved in GABA degradation induced abnormal behaviors in the recipient mice.

369   Importantly, the level of GABA in intestinal content of *S. vestibularis*-treated mice

370   significantly decreased compared with saline-treated mice, which is in accordance

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371   with the inferred GBM and GABA degradation. The schizophrenia-enriched *S.*

372   *vestibularis* contributed to the expression of two types of schizophrenia-relevant

373   behaviors (hyperactivity and impaired social behaviors) in mice. To the best of our

374   knowledge, this is the first study which aims to determine the functional roles of a

375   single bacterium associated with schizophrenia in disease pathogenesis. Although the

376   biological mechanisms underlying the effects of S. vestibularis are still unclear, our

377   data indicate profound influences of this microbe on brain neurotransmitters. In

378   conclusion, our study identified a number of schizophrenia-associated bacterial

379   species representing potential microbial targets for future treatment and GBMs of

380   which some may support the synthesis of new microbial metabolites of importance for

381   the development of schizophrenia

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Acknowledgements

Author contribution

X. M., H. J., R. G., F. Z., and Z. J. conceived the study. F. Z., Z. Y., F. Z., L. Y, B. Z., and Q.M. performed mice experiments. W. W., Q. M., Y. F., L. G., Y. D., Y. C., C. C., C. G., and X.M. recruited volunteers and collected samples for the study. W. W., Q. M., Y. F., Z. Y., L. Y., and F. Z. collected the human fecal and blood samples. Z. Y., L. Y, and F. Z. analyzed the human serum. R. G.,Y. J., Q. W., Q. S., Y. X, and B. L. performed bioinformatics analyses. K. K., S. B., L. M, and B. L. advised on the mice experiments. F. Z., R. G.,H. J., Y. J., and Q. W. interpreted the results and wrote the manuscript with extensive revision performed by L.M., S.B., K.K and Y.X. All authors contributed to the final revision of the manuscript.

Competing interests

Authors declare no competing interests.

Data availability

Metagenomic sequencing data for all samples have been deposited in the European Nucleotide Archive (ENA) database under accession identification code ERP111403.

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410   **Figure captions**

411   **Figure 1. Network of mOTUs differentially enriched in healthy controls and**

412   **schizophrenic patients.**

413   Node sizes reflect the mean abundance of mOTUs. mOTUs annotated to species are colored

414   according to family (Red edges, Spearman's rank correlation coefficient > 0.3, P < 0.05; blue

415   edges, Spearman's rank correlation coefficient < −0.3, P < 0.05;).

416   **Figure 2. The gut-brain modules present in schizophrenia-associated bacterial species.**

417   A green dot indicates a statistically significant association between a gut-brain modules

418   present in schizophrenia-associated bacterial species and a metabolite. No dot represents a

419   non-significant association or a non-existent association. The difference in relation to

420   presence between schizophrenic patients and heathy controls was calculated (Chi-square test,

421   P < 0.05). The bar plot shows the frequency of each bacterial species present in schizophrenic

422   patients (SCZ, blue bar) and healthy controls (HC red bar), respectively.

423   **Figure 3. Gut microbiome-based dicrimination between schizophrenic patients and**

424   **healthy controls.**

425   (a) Receiver operating curves (ROC) according to 171 samples of the discovery set (green

426   line) and 90 independent validation samples (pink line) calculated by cross-validated random

427   forest models. Area under ROC (AUCs) and the 95% confidence intervals (in parentheses) are

428   also shown. (b) The 26 mOTUs with most weight to discriminate schizophrenic (SCZ)

429   patients and healthy controls (HC) were selected by the cross-validated random forest models.

430   The length of line indicates the contribution of the biomarkers to the discriminative model.

431   The color of each mOTU indicates its enrichment in schizophrenic patients (blue) or healthy

432   controls (purple) or no significant direction (black), respectively. (c) Spearman's correlation

433   of 26 mOTUs classifiers with three types of neurotransmitter in serum (green), seven types of

434   cognitive function evaluated using the MATRICS Consensus Cognitive Battery (purple), and

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435   with the positive score and the negative score of the positive and negative syndrome scale

436   (blue). Only significant associations are displayed with correlation coefficient (P value <

437   0.05). (d). The relative abundance ($\log_{10}$) of 26 mOTU classifiers in 90 HCs and 38 SCZ

438   patients at baseline and on a follow-up (3 months later). GABA: 4-aminobutyric acid; TMT:

439   Trail Making Test; BACS SC: Brief Assessment of Cognition in Schizophrenia; Fluency:

440   Category Fluency in Animal Naming; WMS-III: Wechsler Memory Scale-Third Edition for

441   working memory; HVLT-R: Hopkins Verbal Learning Test-Revised for visual learning; NAB:

442   Neuropsychological Assessment Battery for reasoning and problem solving; MSCEIT: Mayer-

443   Salovey-Caruso Emotional Intelligence Test for social cognition.

444   **Figure 4.** *Streptococcus vestibularis* **induces hyperkinetic behavior and impaired social**

445   **interaction in mice.**

446   (a) Schematic diagram of bacterial transplantation and behavioral test. The gut microbiota of

447   mice was depleted by antibiotics, and then *Streptococcus vestibularis*, *Streptococcus*

448   *thermophilus*, or their storage buffer (saline) was administered via oral gavage and drinking

449   water. A series of behavioral tests were carried out after administration of bacteria. The data

450   are representative of two independent experiments. Open field test (OFT), three-chamber

451   social test (TCST), elevated plus maze (EPM), Barnes maze (BM), and the tail suspension test

452   (TST) (for a detailed description, please consult Supplementary Figure 6). (b) The cumulative

453   distance (meters) in different zones in 30-minute OFT in the three groups of mice (in

454   periphery: $F_{(2,46)} = 11.12$, $P = 0.0001$; in center: $F_{(2,46)} = 9.221$, $P = 0.0004$; total distance: $F_{(2,46)}$

455    $= 13.69$, $P < 0.0001$). (c) the cumulative distance (meters) in every 10-minute time interval of

456   OFT traveled by *Streptococcus vestibularis*-treated mice compared to that traveled by

457   *Streptococcus thermophilus*-treated mice and control mice. Time effect: $F_{(2,92)} = 9.816$, $P =$

458   0.0001; group effect: $F_{(2,46)} = 13.69$, $P < 0.0001$; time × group effect: $F_{(4,92)} = 1.808$, $P = 0.134$.

459   (d) The number of rearing in *Streptococcus vestibularis*-treated mice compared with

460   *Streptococcus thermophilus*-treated mice and saline-treated mice during a 30-minute OFT (P

461    $= 0.017$). (e-g) TCST comparing sociability of *Streptococcus vestibularis*-treated mice to that

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462   of *Streptococcus thermophilus*-treated mice and control mice. The results show that

463   *Streptococcus thermophilus*-treated mice and saline-treated mice display obvious sociability,

464   i.e. demonstrate an increase in the number of times of probing a mouse (e, $P < 0.0001$) and

465   spend longer time interacting with a mouse (f, $P = 0.002$) compared to an empty cage, and

466   obvious social novelty, i.e. spend longer time interacting with an unacquainted mouse (new

467   mouse; g, $P = 0.005$, DF = 90, t = 4.277) in comparison with an acquainted mouse. However,

468   these types of social behaviors were not observed in *Streptococcus vestibularis*-treated mice

469   (for sociability : e, $P = 0.881$, DF = 90, t = 0.665; f, $P = 0.282$, DF = 90, t = 1.639; for social

470   novelty, g, $P = 0.923$; DF = 90, t = 0.564). Data are presented as Means ± SEM (n = 15～6

471   per group). The circle represents one value from individual mice (b and c). *P* values were

472   determined one-way analysis of variance (ANOVA) (b), repeated measure two-way ANOVA

473   followed by Sidak's multiple comparisons test (c), Kruskal-Wallis test followed by Dunn's

474   multiple comparisons test (d), or two-way (ANOVA) followed by Sidak's multiple

475   comparisons test (e to g). Blue *P* value indicates the comparison between the *Streptococcus*

476   *vestibularis*-treated and the saline-treated mice and green *P* value indicates the comparison

477   between *Streptococcus thermophilus*-treated and saline-treated mice (b, c).

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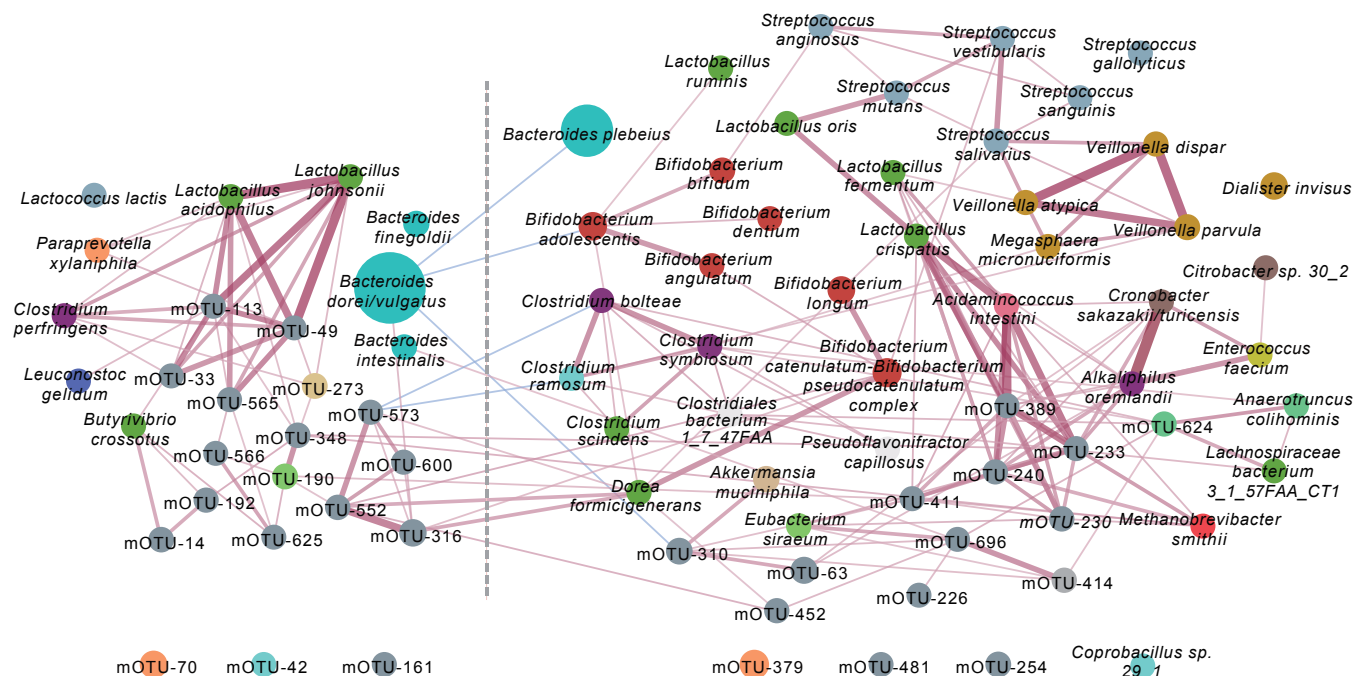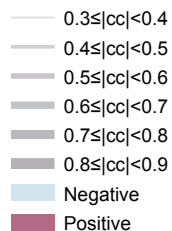637 *Molecular psychiatry* (2019).

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Healthy controls-enriched mOTUs

Schizophrenic patients-enriched mOTUs

Spearman correlation coefficient (cc)

- 0.3≤|cc|<0.4
- 0.4≤|cc|<0.5
- 0.5≤|cc|<0.6
- 0.6≤|cc|<0.7
- 0.7≤|cc|<0.8
- 0.8≤|cc|<0.9
- Negative
- Positive

Actinobacteria
- *Bifidobacteriaceae*

Bacteroidetes
- *Bacteroidaceae*
- *Prevotellaceae*
- *Rikenellaceae*

Euryarchaeota
- *Methanobacteriaceae*

Firmicutes
- *Eubacteriaceae*
- *Lactobacillaceae*
- *Leuconostocaceae*
- *Streptococcaceae*
- *Veillonellaceae*
- *Enterococcaceae*
- *Erysipelotrichaceae*

- *Clostridiaceae*
- *Ruminococcaceae*
- *Acidaminococcaceae*

Verrucomicrobia
- *Verrucomicrobiaceae*
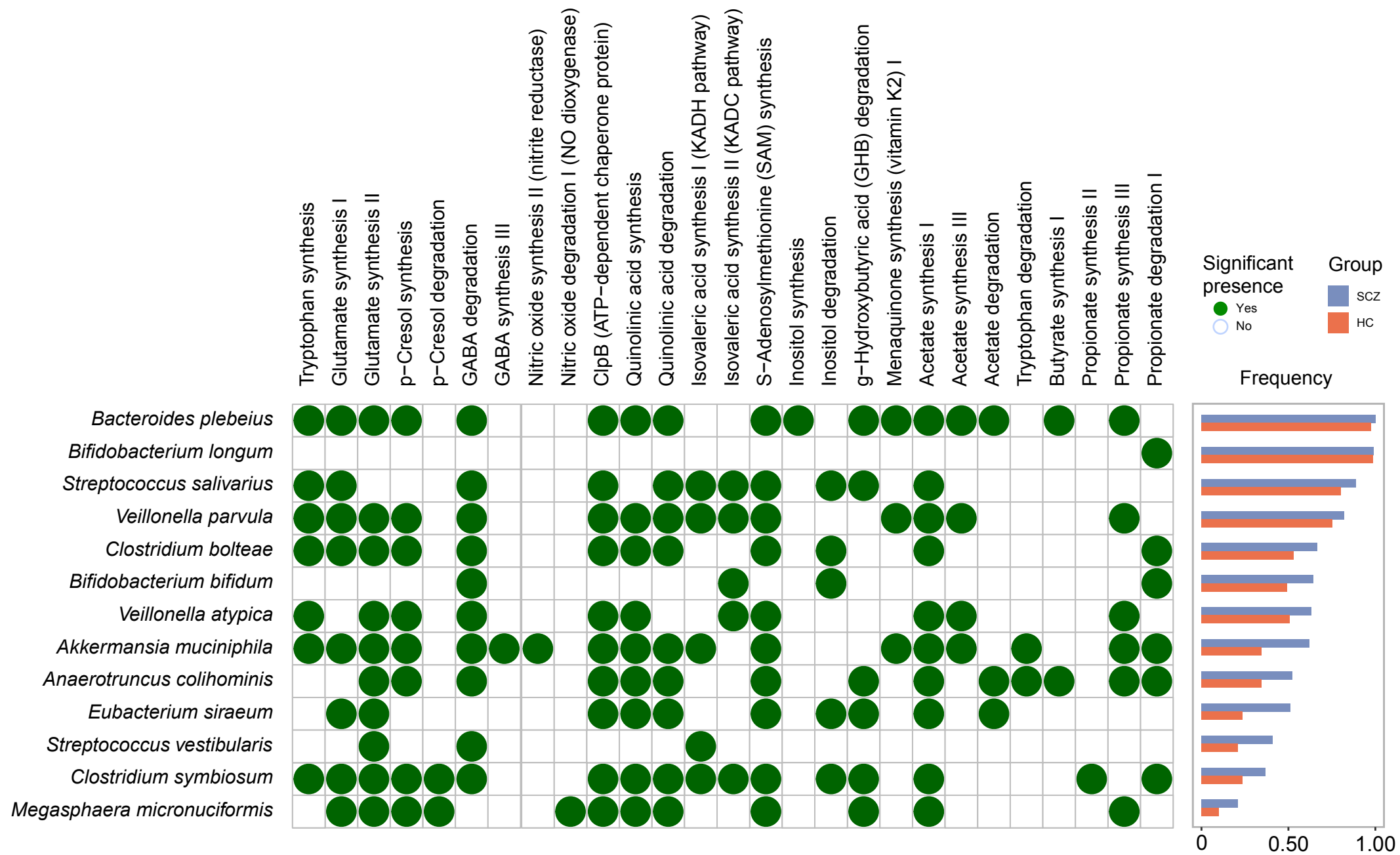
Proteobacteria
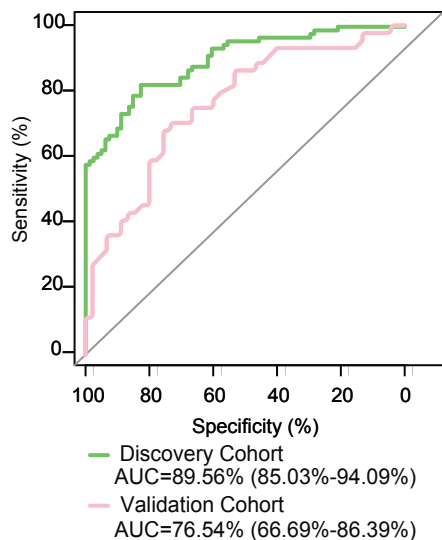- *Enterobacteriaceae*
- *Sutterellaceae*
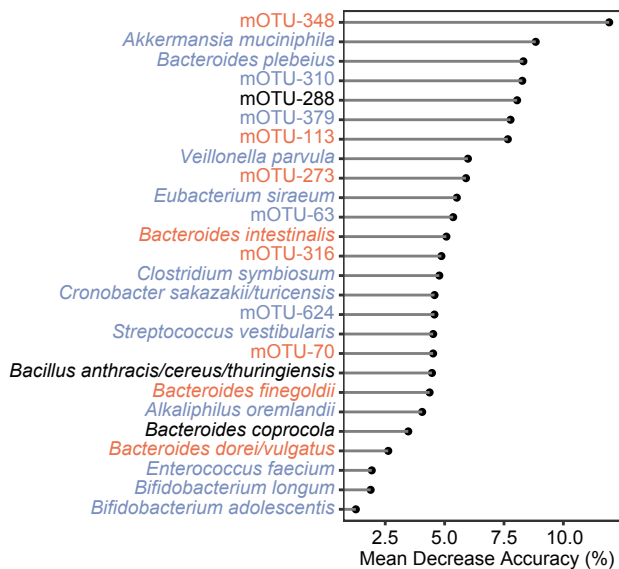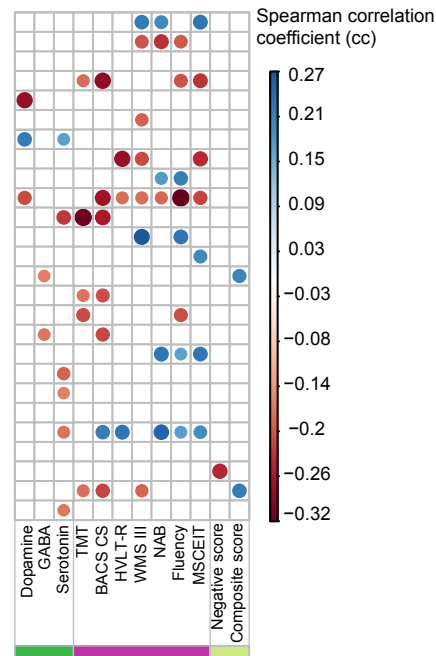
Unclassified
- Unclassified family
- Unclassified mOTU

a

OFT EPM BM TCST TST

0 3 14 25 35 Days

Antibiotics

Treatment of
S. vestibularis and S. thermophilus

Behavioral Tests

b

Distance traveled (m)

P = 0.402
P = 0.002
P = 0.121
P = 0.001
P = 0.094
P < 0.001

Center Periphery Total

Saline
S. vestibularis
S. thermophilus

c

Distance traveled (m) per 10 min

P = 0.306
P = 0.031
P = 0.758
P = 0.0002
P = 0.8595
P < 0.0001

0~10 min 10~20 min 20~30 min

Time

Saline
S. vestibularis
S. thermophilus

d

Number of rears

P > 0.999
P = 0.0256

Saline *S. vestibularis* *S. thermophilus*

e

Total times of probe

P < 0.0001
P = 0.881
P = 0.032

Empty Cage Mouse Empty Cage Mouse Empty Cage Mouse

Saline
S. vestibularis
S. thermophilus

f

Interaction time (s)

P < 0.0001
P = 0.282
P = 0.007

Empty Cage Mouse Empty Cage Mouse Empty Cage Mouse

Saline
S. vestibularis
S. thermophilus

g

Total times of probe

P = 0.0001
P = 0.923
P = 0.001

Old mouse New mouse Old mouse New mouse Old mouse New mouse

Saline
S. vestibularis
S. thermophilus