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1 **Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and**
2 **depressive-like behaviour in a mouse model of metabolic syndrome**

3

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Supplementary Methods

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32 *Culture dependent microbial analysis*

33 Pooled fresh faecal samples collected from each cage of mice were analysed in duplicate following 2 and 5
34 weeks of intervention. Microbial analysis involved enumeration of *L. brevis* DSM32386 and *L. brevis* DPC6108
35 strains after plating serial dilutions on MRS agar supplemented with 100 µg rifampicin/mL (Sigma-Aldrich
36 Ireland Ltd.) and incubating anaerobically for 48 h at 37°C. In addition, isolated colonies were tested for GABA
37 production *in vitro*, as described previously¹. Briefly, isolated colonies were grown anaerobically in MRS
38 containing 3% (w/v) and 1% (w/v) MSG at 37°C for 55 h. Samples were then deproteinized by mixing equal
39 volumes of 24% (w/v) trichloroacetic acid (TCA) and culture, allowed to stand for 10 min and centrifuged at
40 14,000g for 10 min. Supernatants were removed and diluted with 0.2 mol/L sodium citrate buffer, pH 2.2 to
41 yield 250 nmol of each amino acid residue. Samples were then diluted with the internal standard, norleucine, to
42 yield a final concentration of 125 nmol/mL. Amino acids were quantified using a Jeol JLC-500/V amino acid
43 analyser (Jeol Ltd, Garden City, Herts, UK) fitted with a Jeol Na⁺ high-performance cation exchange column.
44 To calculate the % bioconversion of 1% MSG to GABA the following calculation was used:
45 $\text{Glutamate in MRS (nmol/mL)} - \text{Glutamate in sample (nmol/mL)} = \text{nmol/mL of MSG consumed}$
46 $(\text{GABA in sample (nmol/mL)} / \text{nmol/mL of MSG consumed}) * 100$

47

48 *Glucose and insulin tolerance tests*

49 After 12 weeks of feeding, an intraperitoneal-glucose tolerance test (IP-GTT) and an intraperitoneal-insulin
50 tolerance test (IP-ITT) was performed in the LFC (*n* 7) and HFC (*n* 7) groups. After 10 weeks of intervention,
51 the IP-GTT and IP-ITT were performed on individual mice in the LFC, HFC, DPC6108 and DSM32386 groups.
52 For the IP-GTT, mice were injected with a glucose load (1g/Kg body weight) directly into the peritoneal cavity,
53 following a 6 h fast. Blood glucose levels were measured before and 15, 30, 60, 90 and 120 min after glucose
54 load. For the IP-ITT, mice were injected with an insulin load (0.75IU/g bodyweight) directly into the peritoneal
55 cavity, following a 6 h fast. Blood glucose levels were measured before and 15, 30, 60, 90 and 120 min after
56 insulin load. The concentration of blood glucose during the IP-GTT and IP-ITT was determined using a glucose
57 meter (Accu-Chek Aviva, Roche Diabetes Care Ltd., West Sussex, UK) on blood samples collected from the tip
58 of the tail vein.

59

60 ***Insulin resistance index***

61 The plasma insulin concentrations were measured in plasma collected from tail blood during the IP-GTT, after
62 10 weeks of intervention, using a Mouse Insulin ELISA kit (Merckodia, Uppsala, Sweden), according to the
63 manufacturer's instructions. The insulin resistance index was determined by multiplying the area under the
64 curve of both the blood glucose (0 to 120 min) and the plasma insulin (0 to 15 min) obtained from the IP-GTT.

65

66 ***Mixed-meal tolerance test***

67 A mixed-meal tolerance test was performed after 10 weeks of intervention. Mice were fasted for 6 h and a
68 baseline blood sample was taken from the tail following tail incision and collected into EDTA tubes (BD
69 Diagnostics). Mice were then administered 200µl of Ensure Plus liquid diet (1.5kcal/mL, 29.5% fat; Abbott
70 Nutrition, Dublin, Ireland) by intragastric gavage. Blood was collected 2, 4 and 18 h post-gavage. Individual
71 blood samples were collected in microtainer™ collection tubes containing ethylenediaminetetraacetic acid
72 (EDTA) (BD Microtainer Plasma Separator Tubes, BD Diagnostics), thoroughly mixed in the tube and stored on
73 ice until centrifugation for 10 min at 2,000g to isolate the plasma. Isolated plasma was immediately transferred
74 to a clean eppendorf tube following centrifugation. The plasma was then analysed for cholesterol concentration
75 at time points T0, T2, T4 and T18 h (EnzyChrom colorimetric assay; Cambridge Biosciences, UK).

76

77 ***Bioinformatic analysis by QIIME***

78 Sequences obtained from Illumina sequencing were processed using Quantitative Insights Into Microbial
79 Ecology (QIIME) software package version 1.9². The paired-end reads were associated to the corresponding
80 sample through the unique barcode and joined. Reads were further processed with the inclusion of quality
81 filtering based on a quality score of > 20 followed by subsequent removal of sequences below length threshold².
82 UCLUST was then used for clustering the reads left into operational taxonomic units (OTUs) at 97% identity³.
83 PyNAST⁴ was used to align OTUs with a minimum alignment of 150 bp and 80% of minimum identity, and
84 taxonomy was assigned by using Ribosomal Database Project (RDP) classifier 2.0.1⁵. QIIME was used to
85 generate alpha (Chao1, observed OTUs) and beta diversities (Bray Curtis) distance matrices, and principal
86 coordinate analysis (PCoA) plots were generated based on the beta diversity distance matrices. The data
87 generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available
88 under Ac. No. PRJNA414526.

89

90 ***Behaviour test battery***

91 For all behavioural tests, mice were habituated to the testing room by placing home-cages in the test room for at
92 least 30 min prior to testing. The same mice were assessed across all behavioural tests. The behaviour tests were
93 completed over two weeks. All apparatus were cleaned with 70% (v/v) ethanol between mice in each test. A
94 researcher remained in the testing room during each behavioural measure. All outputs were measured by an
95 experimenter blinded to the experimental groups.

96

97 ***Aversive open field test***

98 Following eight weeks of dietary intervention, mice were tested in the open field (OF) for anxiety-related
99 behaviour and locomotor activity. The apparatus was a grey plastic open arena without any bedding (40 cm × 30
100 cm × 25 cm, *L* × *W* × *H*). At the beginning of each trial, mice were placed in the centre of the brightly
101 illuminated (1,000 lux) open field arena. Mice were allowed 10 min free exploration in the box. During this
102 time, behaviour was recorded using a video camera and the number of faecal pellets in the arena were counted
103 as an index of anxiety. At the end of each trial, mice were returned to their home cages with littermates. Total
104 activity and time spent in inner zone were analysed using a tracking system from recorded material (Ethovision,
105 Noldus, Wageningen, The Netherlands).

106

107 ***Novel object recognition***

108 Following eight weeks of dietary intervention, the novel object recognition (NOR) test was used to evaluate
109 cognition (memory and learning) and was conducted as previously described^{6,7}. Day 1, the habituation phase,
110 was performed as the OF test (as described above) where no objects were placed in the grey plastic open arena
111 (40 cm × 30 cm × 25 cm, *L* × *W* × *H*) under low light conditions (60 lux). Day 2, 24 h following the
112 habituation/open field test, mice were reintroduced to the arena containing two identical objects placed in
113 adjacent corners of the arena, approximately 5 cm from each wall. Mice were again allowed 10 min free
114 exploration in the arena, during such time, behaviour was recorded using a video camera as above. Day 3, 24 h
115 after day 2, mice were once again reintroduced to the arena, this time containing one familiar and one novel
116 object, and again, mice were allowed free exploration of the arena for 10 min and during this time, behaviour
117 was recorded using a video camera. After each phase mice were returned to their home cages with littermates.

118 The arena and objects were cleaned with 70% (v/v) ethanol between trials. Object exploration was defined as
119 the time when the animal's nose comes within a 2-cm radius of the object. Memory was defined by the
120 discrimination index for the novel object (DI) as the difference of time mice spent investigating between the
121 novel and the familiar object divided by the total time exploring both objects. [Discrimination Index, DI =
122 (Novel Object Exploration time - Familiar Object Exploration time) / (Novel Object Exploration time + Familiar
123 Object Exploration time)].

124

125 *Elevated plus maze*

126 Following nine weeks of dietary intervention, mice were tested in the elevated plus maze (EPM) test to assess
127 anxiety-like behaviour. The apparatus used was an elevated (1 m from the floor) cross plastic maze, comprising
128 two closed 'safe' arms and two open 'fearful' arms (50 cm × 5 cm × 15 cm walls). Mice were individually
129 placed into the centre of the maze facing an open arm to avoid direct entrance into a closed arm and left to
130 explore for five minutes. Both the time spent in each arm, as well as the number of entries was scored manually
131 (entrance in one arm being defined as all four paws inside the arm). At the end of each trial, mice were returned
132 to their home cages with littermates.

133

134 *Forced swim test*

135 Following nine weeks of dietary intervention, depressive-like behaviour and stress responsiveness were assessed
136 using the forced swim test (FST), as previously described ⁸. Mice were individually placed in a transparent
137 plexi-glass cylinder (24 cm x 21 cm, $H \times D$), containing 15 cm-depth water maintained at room temperature (22
138 $\pm 1^\circ\text{C}$) for a single six minute trial. Water was renewed between each trial. The total time of immobility was
139 scored in the last four minutes ⁹. Immobility was defined as the total absence of movement, except slight
140 motions to maintain the head above water. After the trial, mice were gently dried and single-housed for two
141 hours of recovery, before being placed back to their home cages with littermates.

142

143 *Stress-induced corticosterone production*

144 To assess stress-responsiveness, blood samples were taken in response to an acute stress (FST). First, a blood
145 sample was collected from the tail following tail incision, five minutes before the test. After the acute stress,
146 mice were singly housed following removal from the FST, and blood samples were collected at 15, 45, 90 and
147 120 minutes after the test.

148 Bleeding was performed in a separate room to the FST. Blood samples (50-70µl) were taken from the
149 tail and collected in heparin coated capillary tubes. The blood was then transferred to a microtainer™ collection
150 tubes containing EDTA (BD Diagnostics), thoroughly mixed in the tube and stored on ice until centrifugation
151 for 10 min at 2,000g to isolate the plasma. Isolated plasma was immediately transferred to a clean eppendorf
152 tube following centrifugation. Isolated plasma was stored at -80 °C for later corticosterone quantification.
153 Corticosterone was quantified using a commercially available ELISA kit (Enzo Life Sciences (UK) Ltd., Exeter,
154 UK) according to the manufacturer's protocol.

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- 156 1 Barrett, E., Ross, R., O'toole, P., Fitzgerald, G. & Stanton, C. γ -Aminobutyric acid production by
157 culturable bacteria from the human intestine. *Journal of applied microbiology* **113**, 411-417 (2012).
158 2 Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nature*
159 *methods* **7**, 335 (2010).
160 3 Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**, 2460-
161 2461 (2010).
162 4 Caporaso, J. G. *et al.* PyNAST: a flexible tool for aligning sequences to a template alignment.
163 *Bioinformatics* **26**, 266-267 (2009).
164 5 Maidak, B. L. *et al.* The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29**, 173-174 (2001).
165 6 Burokas, A., Gutiérrez-Cuesta, J., Martín-García, E. & Maldonado, R. Operant model of frustrated
166 expected reward in mice. *Addiction biology* **17**, 770-782 (2012).
167 7 Pusceddu, M. M. *et al.* N-3 Polyunsaturated Fatty Acids (PUFAs) Reverse the Impact of Early-Life
168 Stress on the Gut Microbiota. *PloS one* **10**, e0139721, doi:10.1371/journal.pone.0139721 (2015).
169 8 Cryan, J. F. *et al.* Use of dopamine- β -hydroxylase-deficient mice to determine the role of
170 norepinephrine in the mechanism of action of antidepressant drugs. *Journal of Pharmacology and*
171 *Experimental Therapeutics* **298**, 651-657 (2001).
172 9 Cryan, J. F., Markou, A. & Lucki, I. Assessing antidepressant activity in rodents: recent developments
173 and future needs. *Trends in pharmacological sciences* **23**, 238-245 (2002).

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Supplementary Tables and Figures:

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187 **Supplementary Table S1: Diet information**

Product #	D15072701		D12492	
	gm	<i>Kcal</i> (%)	gm	<i>Kcal</i> (%)
Protein	19	20	26	20
Carbohydrate	67	70	26	20
Fat	4	10	35	60
Total		100		100
kcal/gm	3.8		5.2	
Ingredient	gm	<i>kcal</i>	gm	<i>kcal</i>
Casein	200	800	200	800
L-Cysteine	3	12	3	12
Corn Starch	280	1120	0	0
Maltodextrin 10	140	560	125	500
Sucrose	280	1120	68.8	275
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	245	2205
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0	0	0	0
FD&C Red Dye #40	0.025	0	0	0
FD&C Blue Dye #1	0.025	0	0.05	0
Total	1055.05	4057	773.85	4057

188 **Supplementary Table S2: Alpha and Beta Diversity Indexes**

	Chao_1	OTUs (n)	Bray-Curtis
LFC	4397 ± 621 ^a	2501 ± 383 ^a	0.36 ± 0.06 ^a
HFC	3599 ± 576 ^b	1943 ± 330 ^b	0.41 ± 0.06 ^b
DPC6108	4138 ± 720 ^a	2238 ± 494 ^{a,b}	0.42 ± 0.09 ^{b,d}
DSM32386	3375 ± 487 ^b	1857 ± 328 ^b	0.44 ± 0.08 ^{c,d}

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193 **Supplementary Table S3: Diet appeared to be the main determinant of microbiota-biochemistry**
 194 **associations**

	Glycaemia_GTT	Glycaemia_ITT	Insulin	Cholesterolemia
<i>Bifidobacteria</i>	↓	↓	-	↓
<i>Parabacteroides</i>	-	↑	↓	↑
<i>Muribaculum</i>	↓	↓	-	↓
<i>Odoribacter</i>	↓	↓	-	-
<i>Bacteroidetes_other</i>	-	-	-	↑
<i>Erysipelotrichaceae</i>	↓	-	-	↓

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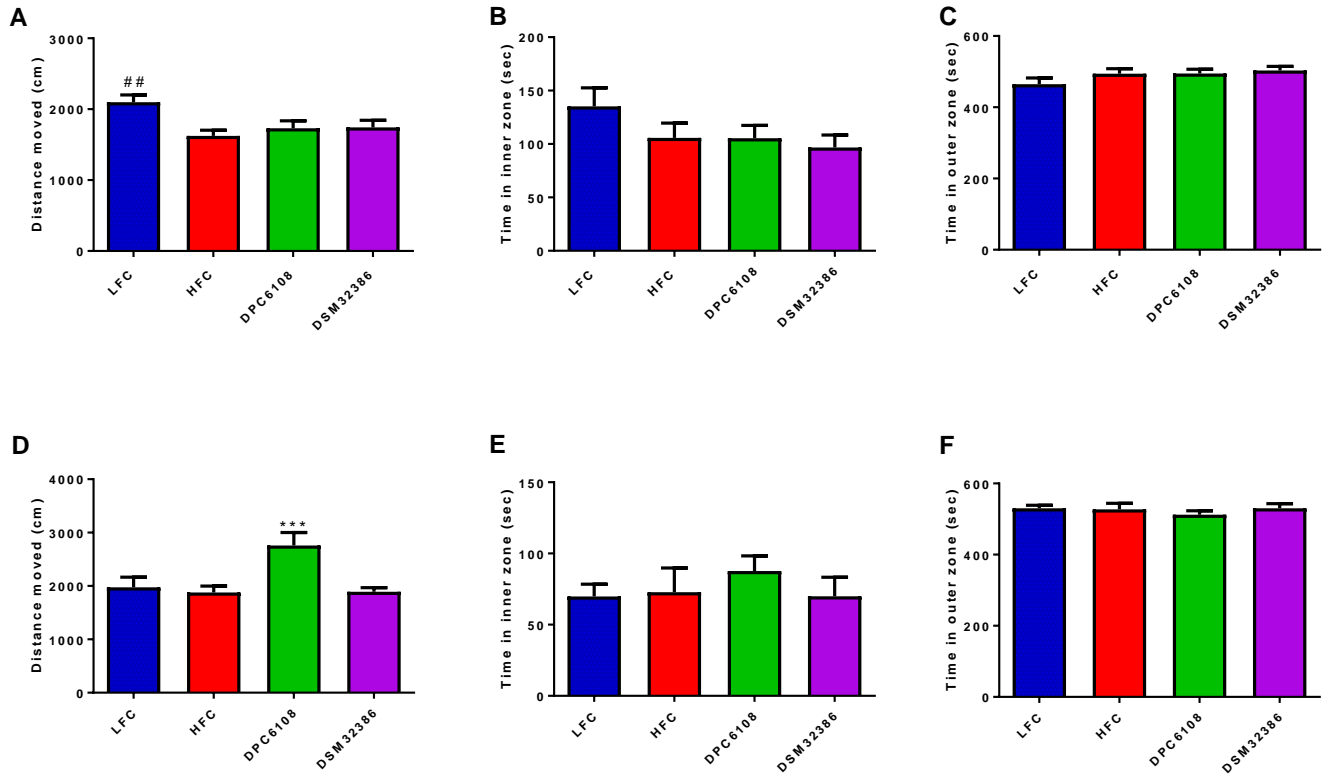
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212 **Supplementary Figure S1: *L. brevis* had no effect on anxiety-like behaviour in the aversive and NOR open**
213 **field test**

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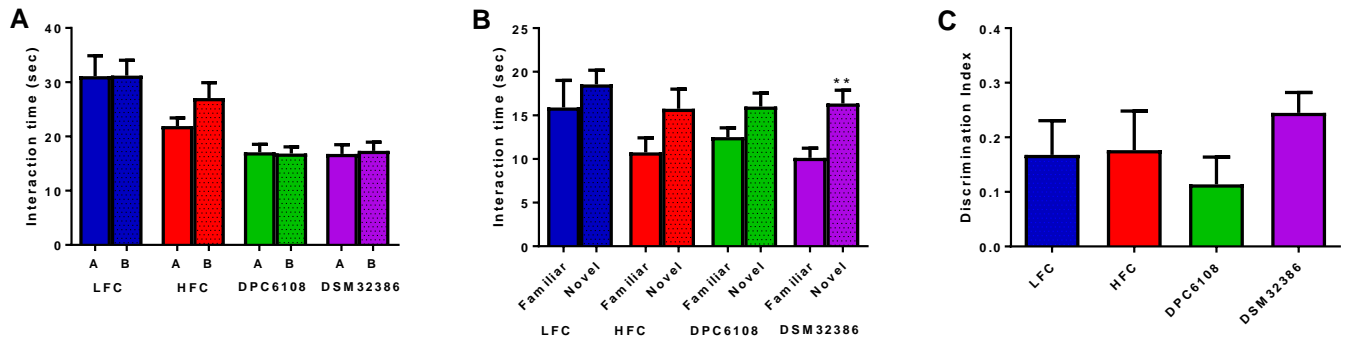
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229 **Supplementary Figure S2: *L. brevis* DSM32386 had a modest effect on object recognition behaviour /**
230 **cognitive function in the NOR test**



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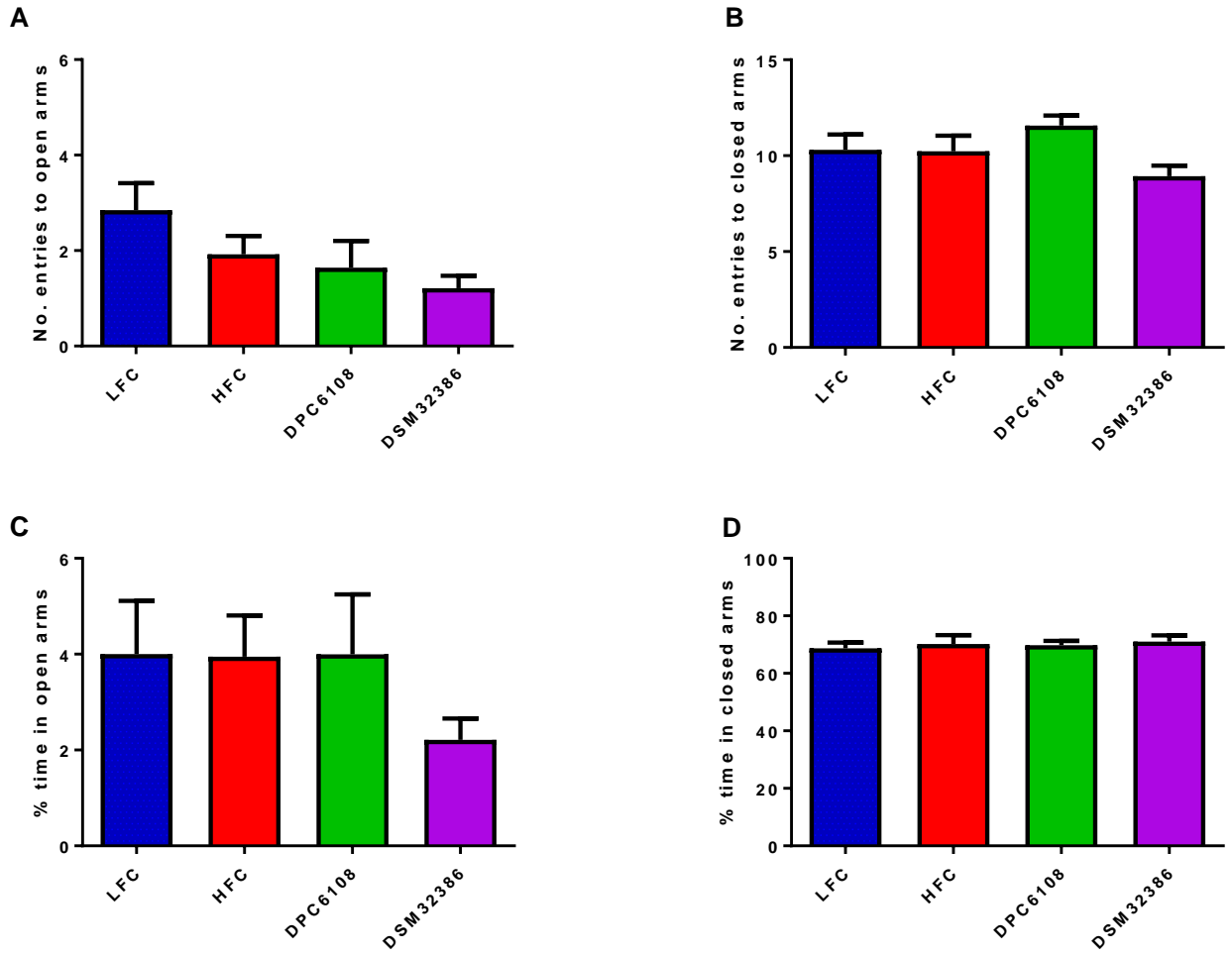
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Supplementary Figure Legends

269

270 **Supplementary Table S1:** Mice were fed *ad libitum* with either a low fat diet (Open Source Diets (D15072701
271 – 10% kcal from fat and equal parts corn starch and sucrose; (Research Diets Inc., NJ 08901 USA)) or a high fat
272 diet (Open Source Diets (D12492 – 60% kcal from fat; Research Diets Inc.)) and were allowed free access to
273 food and water, for 24 weeks.

274 **Supplementary Table S2:** Alpha (Chao-1, numbers of observed OTUs) and Beta (Bray-Curtis) diversity
275 indexes are shown as mean values \pm standard deviation. Different letters indicate a significant difference (p
276 <0.05).

277 **Supplementary Table S3: Diet appeared to be the main determinant of microbiota-biochemistry**
278 **associations.** Correlation between the faecal microbiota and glycaemia during IP-GTT, glycaemia during IP-
279 ITT, insulin or cholesterol levels in the blood. Green arrows indicate a negative correlation, while red arrows
280 indicate a positive correlation ($p <0.05$). The dash indicates no significant correlation ($p >0.05$).

281 **Supplementary Figure S1: *L. brevis* had no effect on anxiety-like behaviour in the aversive and NOR open**
282 **field test.** The effect of HF-diet feeding and probiotic interventions on anxiety-like behaviour was assessed after
283 21 weeks of feeding and after 8 weeks of intervention. Total distance moved (**A**), time spent in the inner zone
284 (**B**) and time spent in the outer zone (**C**) was measured in the aversive OF test and the same outcomes were
285 again measured in the NOR OF test (**D, E and F, respectively**) for LFC (n=13), HFC (n=13), DPC6108 (n=14),
286 DSM32386 (n=14). Data are expressed as mean \pm SEM. All data was analysed using the appropriate unpaired
287 student t-test (HFC vs LFC) and one-way analysis of variance (ANOVA). ## $p < 0.01$ HFC vs LFC, * $p < 0.05$
288 treatment vs HFC. HF: high fat, HFC: high fat control, DPC6108: *L. brevis* DPC6108, DSM32386: *L. brevis*
289 DSM32386, LFC: low fat control.

290 **Supplementary Figure S2: *L. brevis* DSM32386 had a modest effect on object recognition behaviour /**
291 **cognitive function in the NOR test.** The effect of HF diet feeding and microbial interventions on cognitive
292 function was assessed after 21 weeks of feeding and 8 weeks of intervention. On day 1 of the test, mice were
293 allowed to familiarise themselves with two identical objects (**A**). On day 2, one of the familiar objects was
294 replaced by a novel object (**B**) and the discrimination index represents how the mice could identify the change
295 between the familiar and novel object during day 2 (**C**). The NOR test was performed on LFC (n=13), HFC

296 (n=13), DPC6108 (n=14), DSM32386 (n=14). Data are expressed as mean \pm SEM. All data was analysed using
297 the appropriate unpaired student t-test (HFC vs LFC) and one-way analysis of variance (ANOVA). ** $p < 0.01$
298 novel vs familiar. NOR: novel object recognition, HF: high fat, HFC: high fat control, DPC6108: *L. brevis*
299 DPC6108, DSM32386: *L. brevis* DSM32386, LFC: low fat control.

300 **Supplementary Figure S3: *L. brevis* had no effect on anxiety-like behaviour in the EPM test.** The effect of
301 HF-diet feeding and microbial interventions on anxiety-like behaviour was assessed after 21 weeks of feeding
302 and after 8 weeks of intervention. The number of entries to the open (**A**) and closed (**B**) arms of the maze was
303 assessed and the percentage of time spent in the open (**C**) and closed (**D**) arms was also calculated for LFC
304 (n=13), HFC (n=13), DPC6108 (n=14), DSM32386 (n=14). Data are expressed as mean \pm SEM. All data was
305 analysed using the appropriate unpaired student t-test (HFC vs LFC) and one-way analysis of variance
306 (ANOVA). EPM: elevated plus maze, HF: high fat, HFC: high fat control, DPC6108: *L. brevis* DPC6108,
307 DSM32386: *L. brevis* DSM32386, LFC: low fat control.