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## Supplementation of dietary non-digestible oligosaccharides from birth onwards improve social and reduce anxiety-like behaviour in male BALB/c mice

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### ABSTRACT

**Objective:** The intestinal microbiota is acknowledged to be essential in brain development and behaviour. Their composition can be modulated by prebiotics such as short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharide (lcFOS). Several studies reported potential health benefit of prebiotics on behaviour. As the prebiotic mixture of scGOS and lcFOS is included in infant formula, we investigated the effects of dietary supplementation with this specific mixture from the day of birth onwards on behaviour and intestinal microbiota development in mice.

**Method:** Healthy male BALB/cByJ mice received, from day of birth, a dietary supplement with or without 3% scGOS:lcFOS (9:1). Behavioural tests were performed pre-weaning, in adolescence, early adulthood and adulthood. We assessed faecal microbiota compositions over time, caecal short-chain fatty acids as well as brain mRNA expression of *Htr1a*, *Htr1b* and *Tph2* and monoamine levels.

**Results:** Compared to control fed mice, scGOS:lcFOS fed mice showed reduced anxiety-like and repetitive behaviour over time and improved social behaviour in adulthood. The serotonergic system in the prefrontal cortex (PFC) and somatosensory cortex (SSC) was affected by the scGOS:lcFOS. In the PFC, mRNA expression of brain-derived neurotrophic factor (*Bdnf*) was enhanced in scGOS:lcFOS fed mice. Although the bacterial diversity of the intestinal microbiota was unaffected by the scGOS:lcFOS diet, microbiota composition differed between the scGOS:lcFOS and the control fed mice over time. Moreover, an increased saccharolytic and decreased proteolytic fermentation activity were observed in caecum content.

**Discussion:** Supplementing the diet with scGOS:lcFOS from the day of birth is associated with reduced anxiety-like and improved social behaviour during the developmental period and later in life, and modulates the composition and activity of the intestinal microbiota in healthy male BALB/c mice. These data provide further evidence of the potential impact of scGOS:lcFOS on behaviour at several developmental stages throughout life and strengthen the insights in the interplay between the developing intestine and brain.

### KEYWORDS

Behaviour; prebiotics; galacto-oligosaccharide; fructo-oligosaccharide; intestinal microbiota; SCFA; healthy mice; dietary supplementation from birth; early life; serotonergic system

## Introduction


The trillions of microbes colonizing our intestine, collectively referred to as the intestinal microbiota, have an essential role in health and disease throughout life [1–3]. The establishment of the intestinal microbiota is influenced by several factors, such as gestational age, delivery-mode and nutrition (e.g. breast- or formula-feeding) and early life antibiotic exposure [4]. These factors critically influence the optimal maturation of the metabolic, immune and neurological systems [4], i.e.

alterations of intestinal microbiota composition (e.g. due to antibiotic-exposure and cesarean section) can increase the risk of developing metabolic-related (e.g. obesity), immune-related (e.g. allergies), but also behaviour-related brain disorders (e.g. autism spectrum disorder) [3,5].

In both human and mice, microbial compositional changes can lead to behavioural changes [1,2,6,7]. The importance of the intestinal microbiota for brain development and behaviour has been demonstrated in

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animals treated with antibiotics or in germ-free animals. Compared with conventional colonized mice, germ-free mice show social deficits and reduced anxiety [1]. In addition, germ-free mice also have altered neurochemical messenger systems [8]. Antibiotic-treated mice show cognitive impairments, reduced anxiety and reduced sociability [7]. Interestingly, behavioural impairments caused by depletion of the microbiota either with antibiotics or in germ-free mice were ameliorated by bacterial re-colonization or treatment with probiotics, indicating that changes in the microbiota are closely connected to behaviour [1,7].

Prebiotics modulate the growth and activity of the intestinal microbiota and several studies reported the capability of prebiotics to also modulate behaviour. The prebiotic mixture of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) (1:1) reduced anxiety-like and depression-like behaviour in adult mice [2]. Moreover, a prebiotic mixture of polydextrose and GOS administered during early life, diminished stress-induced behaviour in juvenile rats, improved recognition memory and increased explorative behaviour in young pigs [9,10].

Human milk is known to contain substrates with prebiotic function, known as human milk oligosaccharides, to specifically shape the intestinal microbiota of newborns [11]. Also, the human milk oligosaccharide 2'-fucosyllactose (2'FL) possess cognitive modulatory capacities; rat pups exposed to 2'FL during lactation showed improved cognition in adulthood [12].

The specific prebiotic mixture of 90% of the low molecular mass short-chain GOS and 10% of the high molecular mass long-chain FOS (scGOS:lcFOS, 9:1) similar to the molecular size distribution of human milk oligosaccharides is included in infant formula and have been shown to exert similar functionalities as human milk oligosaccharides [11]. These prebiotic carbohydrates can be digested by specific bacteria, such as *Bifidobacterium* and *Bacteroides* spp. [4], and subsequently fermented into short-chain fatty acids (SCFAs), which are reported to have neuroactive properties locally and systemically [5,11]. As the behavioural modulatory effects of this specific mixture of scGOS:lcFOS (9:1) is not yet known, we investigated, from the day of birth, in healthy male BALB/c mice, the effects of this specific prebiotics mixture scGOS:lcFOS (9:1) on behaviour and intestinal microbiota development, both in taxonomic and in SCFA-composition.

We showed that dietary supplementation with scGOS:lcFOS from birth in healthy male BALB/c mice reduced anxiety-like and stereotypic behaviour over time and improved social behaviour in adulthood. These behavioural improvements were associated with marked

differences in microbiota composition and microbiota metabolite composition. In addition, the improved behaviour induced by scGOS:lcFOS was accompanied by altered monoamine levels, mRNA expression of serotonergic markers and BDNF mainly in the prefrontal cortex (PFC).

## Material and methods

### Animals

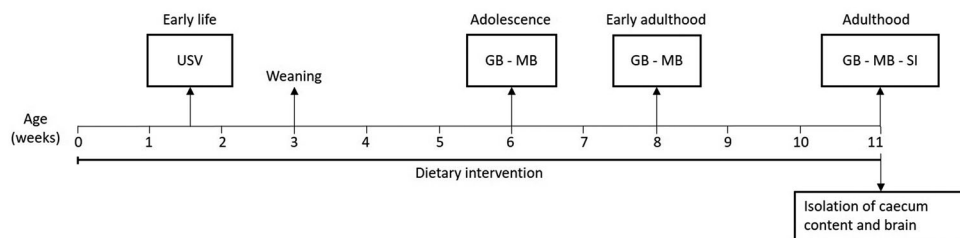
Sixteen days pregnant BALB/cByJ mice were purchased from Charles River Laboratories (Maastricht, The Netherlands). From the day of birth of the litter (post-natal day zero, PND0) the dams were allocated to either the control ( $n = 6$ , pups  $n = 17$ ) or the 3% scGOS:lcFOS ( $n = 5$ , pups  $n = 14$ ) enriched diet. After weaning on PND21, the male offspring ( $n = 10$  per group,  $n = 1-3$  from each litter) continued the same diet as allocated to their mother. Additional male offspring ( $n = 9$ ) from control fed dams were used as interaction mice in the social interaction test (more details in the supplement). All animal experimental procedures were carried out in compliance with national legislation following the EU-Directive for the protection of animals used for scientific purposes, and were approved by the Ethical Committee for Animal Research.

### Diets

Based on the standard AIN-93G control diet, the enriched diet consisted of a 3% (w/w) mixture of short-chain galacto-oligosaccharides (scGOS) (degree of polymerization 2-8) and long-chain fructo-oligosaccharides (lcFOS) (degree of polymerization on average  $\geq 23$ ) in a 9:1 (w/w) ratio added in an isocaloric manner (table S1). Both diets were obtained from Research Diet Services (Wijk bij Duurstede, The Netherlands).

### Experimental design

Figure 1 shows the experimental design. At several ages across lifespan, behavioural tests for anxiety, stereotypic and social behaviour were conducted. In the pre-weaning period, ultrasonic distress vocalization of the male offspring was measured (1.5 weeks old). During adolescence (6 weeks old) and early adulthood (8 weeks old), the marble burying test and self-grooming behaviour were assessed. During adulthood (11 weeks old) the social interaction test was conducted in addition to the marble burying and the self-grooming test. Faecal pellets were collected at 4, 6, 8 and 11 weeks of age and from the dams before starting dietary supplementation. The mice were euthanized by decapitation to collect caecum and



**Figure 1.** Schematic overview of the experimental protocol and the conducted behavioural tests. The mice received either a control diet or a diet enriched with 3% scGOS:lcFOS (9:1) from the day of birth. The ultrasonic distress vocalization test was conducted during infancy. At 3 weeks of age mice were weaned and the male mice continued the allocated diet of the dam. Grooming behaviour and the marble burying test were performed during adolescence, early adulthood and adulthood and the social interaction test was performed during adulthood. After the last behavioural test organs were collected. USV: ultrasonic distress vocalisation. GB: Grooming behaviour. MB: Marble burying test. SI: Social interaction test.

brain. Due to limited brain material of the PFC, amygdala and hippocampus  $n = 5$  was used to measure monoamine levels and  $n = 5$  for qPCR.

### Behavioural tests

Ultrasonic distress vocalization (USV) of male offspring was measured to determine anxiety-like behaviour as described previously [13]. The marble burying test assesses anxiety-like and stereotypic/repetitive behaviour [14]. Mice were scored for spontaneous self-grooming behaviour as previously described [15]. The sociability of the mice was assessed with a previously described social interaction test [15]. Time spent in the interaction zone near the cage of the unfamiliar target mouse; latency to first approach of the interaction zone and total distance moved were analysed (more details in the supplement).

### Measurement of monoamine levels

The pre-frontal cortex (PFC), amygdala (AM), dorsal hippocampus (DH) and the somatosensory cortex (SSC) were, with the previously described HPLC method [16], measured for levels of tryptophan, serotonin (5-hydroxytryptamin, 5-HT) and its metabolite 5-hydroxyindolacetic acid (5-HIAA), noradrenalin (NA), dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA) (details in the supplement). The concentrations of each compound were calculated by comparison with both external and internal standards.

### RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated from PFC, SSC, amygdala and hippocampus using the RNeasy isolation kit (Qiagen) and cDNA was synthesised using iScript cDNA synthesis kit (BioRad) following manufacturer's protocol.

Quantitative real-time PCR was performed on a CFX96 real-time PCR detection system using iQ SYBR green supermix (BioRad) and primers (Qiagen) for *Rps13* (house-keeping gene), 5-hydroxytryptamin 1a receptor (*Htr1a*), *Htr1b*, brain-derived neurotrophic factor (*Bdnf*) and tryptophan hydroxylase 2 (*Tph2*) (details in the supplement). The mRNA expression of the gene of interest was normalized to the housekeeping gene and data are presented as fold change in expression compared to control mice.

### Short-chain fatty acids (SCFAs) levels in caecum content

The levels of short-chain fatty acids (SCFAs): acetic (AA), propionic (PA), butyric (BA), and valeric acid (VA), as well as the branched short-chain fatty acids; isobutyric (iBA) and isovaleric (iVA) acid in the caecum content were quantified with gas chromatography, as described previously [17] (details in the supplement).

### Bacterial DNA extraction from faecal pellets

Total DNA was extracted from faecal pellets. The pellets were mixed with 350  $\mu$ L S.T.A.R. buffer (Roche, Basel, Switzerland), followed by three 1-minute rounds of bead beating (25 g of 0.1 mm zirconia beads plus five 2.5 mm glass beads) on a FastPrep instrument (MP Bio-medicals, Santa Ana, California, USA) at a power level of 5.5. The sample was heated to 95°C and mixed by shaking at 100 rpm for 15 min. The homogenate was centrifuged at 4°C for 5 min at 14,000 g to pellet stool particles. The supernatant, containing DNA, was collected and the pellet was subjected to another extraction round with half the volume of S.T.A.R. buffer. Supernatants were pooled per sample and mixed, and 250  $\mu$ L was further purified on the Maxwell 16 MDx instrument using the cartridge preparation from the Maxwell 16

tissue LEV total RNA purification kit as described by the manufacturer (Promega, Madison, WI, USA). DNA quality and quantity were assessed with the NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, Massachusetts, USA). DNA extracts were diluted to the same concentration before preparation for 16S rRNA-gene sequencing.

### **Gut microbiota profiling by 16S rRNA-gene sequencing**

Extracted faecal DNA was used for microbiota profiling by 16S rRNA-gene sequencing. The V3-V4 region of the 16S rRNA-gene was PCR-amplified with universal primers S-D-Bact-0341-b-S-17 primer (forward 5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 primer (reverse 5'-GACTACHVGGGTATCTAATCC-3') [18] and barcoded using a two-step PCR protocol as described by Van den Bogert et al. [19]. Sequencing was performed on an Illumina HiSeq sequencing platform (Illumina, USA) in a 300 bp paired-end mode.

### **Bioinformatic and analysis of 16S rRNA sequencing data**

The 'Quantitative Insights Into Microbial Ecology' (QIIME) v1.9.0 package was used to analyse sequence data [20]. Settings for demultiplexing and merging of paired-end sequences were as recommended by QIIME (according [http://qiime.org/tutorials/processing\\_illumina\\_data.html](http://qiime.org/tutorials/processing_illumina_data.html)). Sequences were clustered into Operational Taxonomic Units (OTUs) based on 97% sequence identity using VSEARCH v2.03 with chimera checking against the RDP gold database [21,22]. Taxonomic assignment was performed using the RDP classifier against the SILVA119 database [23]. The species diversity ( $\alpha$ -diversity) was calculated using the Chao1 and Shannon index for diversity [24] with correction for the differences in sequencing depths (number of reads per sample) by rarefaction.

### **Statistical analysis**

Statistical analyses were performed comparing the control diet group and the scGOS:lcFOS diet group. Fisher's exact test was performed to determine whether the number of pups emitting calls during the USV test differed between the control diet group and the scGOS:lcFOS diet group. The USV, grooming, marble burying and social interaction tests were analysed with linear mixed models, controlled for litter effect and post hoc Sidak's multiple comparison test, repeated measures were added in the analysis of grooming and marble burying

tests. All other data were statistically analysed with an unpaired, two-tailed Student's *t*-test. When not normally distributed or unequal variances, data were transformed taking the common logarithm before statistical analysis. The Mann-Whitney test was used to analyse differences between the dietary groups for latency of first approach to the target mouse during the social interaction test as the variance was still unequal after transformation. Correlations were analysed using the Spearman correlation. Results were considered statistically significant when  $P < 0.05$ . Analyses were performed using SPSS version 24 and GraphPad Prism Software version 7.03 for Windows (GraphPad Prism software, La Jolla, CA, USA).

### **16S rRNA-gene sequencing**

The species richness and diversity ( $\alpha$ -diversity) indexes calculated in QIIME from the 16S rRNA-gene sequencing data were analysed at one single rarefied sequencing depth. Differences between treatment groups over time were tested by two-way ANOVA with Sidak's multiple comparison test using GraphPad Prism version 7.03 for Windows.

The non-rarefied OTU tables obtained from QIIME were aggregated at the bacterial genus level. Genera present in less than 30% of the samples or with an average relative abundance less than 0.005% were omitted from the analysis. Statistical comparisons were performed by applying a combination of multivariate analysis with Canoco 5 software [25], followed by differential abundance testing using the R-package MetagenomeSeq [26]. Firstly, the constrained ordination method, principal response curves (PRC), was used to test time-dependent treatment effects [27]. The Monte Carlo Permutation test (MCPT), with 1000 permutations, was used to evaluate statistical significance ( $P < 0.05$ ) of the resulting model. Next, the top 10 responding bacterial taxa identified from the model were evaluated on differential abundances at the different timepoints by metagenomeSeq using the zero-inflation log-normal distribution (FitFeatureModel) as recommended by the developers of the package [28]. The Benjamini-Hochberg false-discovery rate (FDR) was used to correct for multiple comparisons in the differential abundance tests [29], and statistical significance was considered for  $FDR < 0.1$  when observed for at least two repeated measurements.

## **Results**

### **Ultrasonic distress vocalization**

Ultrasonic distress vocalization was measured 10 days after birth. The number of male pups emitting at least

one call was not significantly different between the dietary groups (table S2,  $P > 0.05$ ). The pups emitting at least one call were used for further analysis of the USV. The total number of calls was significantly reduced in pups from lactating dams receiving the scGOS:lcFOS diet ( $P < 0.05$ ; Figure 2(A)). The average frequency (Hz) showed a trend towards a decrease in the pups from scGOS:lcFOS diet fed dams ( $P < 0.06$ ) and the average time of a call was not significantly different, figure S1.

### Self-grooming and marble burying

The grooming frequency trended towards an overall effect by diet (control vs scGOS:lcFOS) ( $F(1, 7.241) = 4.055$ ,  $P = 0.083$ ) and overall age was significantly affected ( $F(2, 18) = 32.667$ ,  $P < 0.000001$ ) (Figure 2(B)). The change in grooming frequency over time was dependent on the interaction effect between diet and age ( $F(2, 18) = 4.347$ ,  $P < 0.05$ ). The grooming frequency increased over time from adolescence to adulthood in control diet fed mice (adolescence vs early adulthood  $P < 0.001$ , adolescence vs. adulthood  $P < 0.000001$ ). A significant increase in grooming frequency was only observed between adolescence and adulthood in the scGOS:lcFOS fed mice ( $P < 0.01$ ). Although no significant differences were observed between the diets in grooming frequency in adolescence and early adulthood, in adulthood the mice receiving the scGOS:lcFOS diet showed a significantly lower grooming frequency compared with mice receiving the control diet ( $P < 0.05$ ). However, the scGOS:lcFOS diet displayed no effect on self-grooming duration neither over time nor at any age (Figure 2(C)).

The number of buried marbles was significantly affected by diet (control vs scGOS:lcFOS) ( $F(1, 13.337) = 39.208$ ,  $P < 0.0001$ ) and age ( $F(2, 18) = 9.64$ ,  $P < 0.001$ ). The number of marbles buried over time was independent on the diet (interaction effect between diet and age ( $F(2, 18) = 2.505$ ,  $P > 0.05$ )). Mice receiving the control diet buried significantly more marbles in early adulthood and adulthood compared to adolescence ( $P < 0.05$ ). The scGOS:lcFOS fed mice buried significantly more marbles in adulthood compared with adolescence ( $P < 0.01$ ). From adolescence and onwards the mice exposed to the scGOS:lcFOS diet demonstrated a significant decrease in the number of buried marbles compared to control mice (adolescence  $P < 0.01$ , early adulthood  $P < 0.001$ , adulthood  $P < 0.0001$ ) (Figure 2(D)).

### Social interaction

The scGOS:lcFOS fed adult mice spent as much time in the interaction zone as the control mice in absence of a

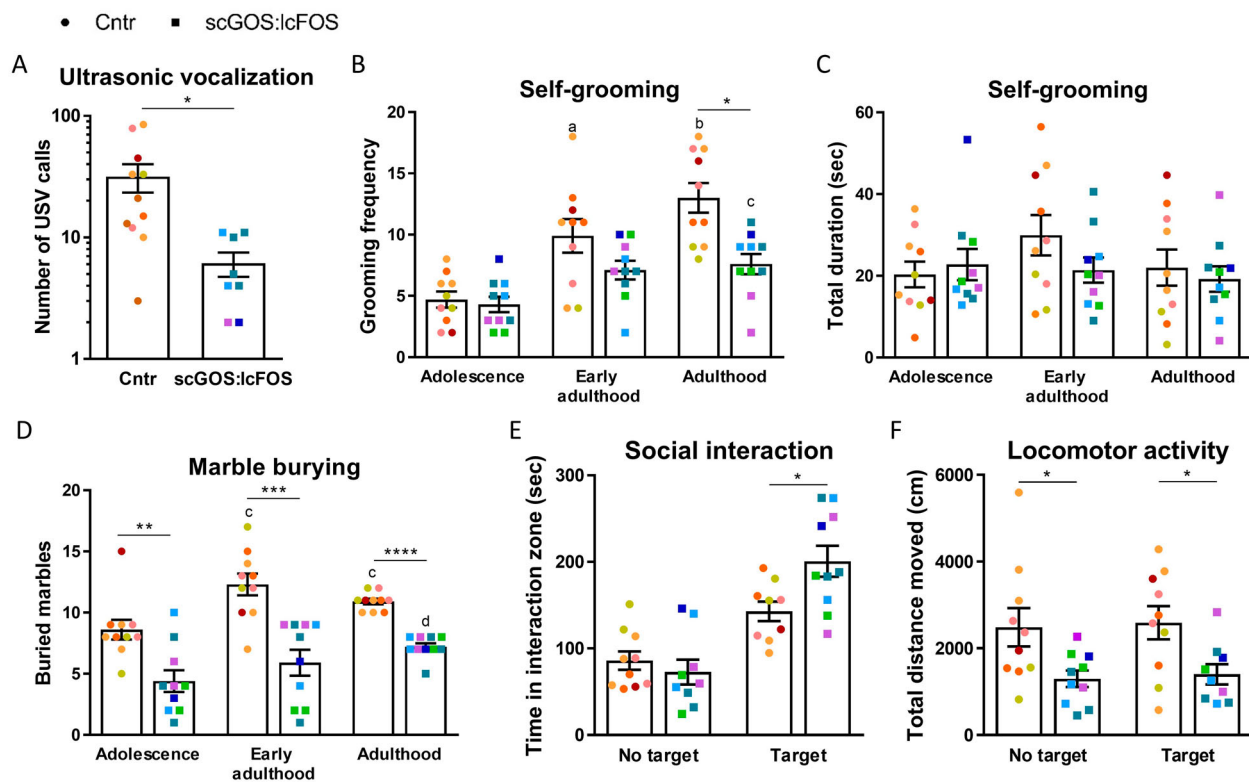
target mouse ( $P > 0.05$ ). However, in presence of a target mouse, mice exposed to the scGOS:lcFOS diet spent significantly more time in the interaction zone than did the control mice ( $P < 0.05$ ) (Figure 2(E)). The latency of first approach to the target mouse and the frequency a mouse entered the interaction zone did not significantly differ between the two dietary groups with either the absence or presence of a target mouse ( $P > 0.05$ ) (figure S1). The locomotion activity measured as total distance moved through the arena was significantly less in the scGOS:lcFOS receiving mice in both absence and presence of a target mouse ( $P < 0.05$ ) (Figure 2(F)).

### Monoamine levels in several brain regions

In the PFC, tryptophan ( $P < 0.05$ ) and 5-HT ( $P < 0.05$ ) levels were significantly decreased in the scGOS:lcFOS dietary group compared with the control group (Figure 3(A)) and the NA, DA and DA metabolites levels were unchanged (figure S2). In the SSC the individual monoamines were unchanged; however, the 5-HT turnover indicated by 5-HIAA/5-HT was significantly higher in the scGOS:lcFOS fed group ( $P < 0.05$ ) (Figure 4(A) and figure S2). The tryptophan and 5-HT levels as well as the 5-HT and the 5HIAA levels correlated significantly in the PFC (Spearman correlation were respectively  $r = 0.782$ ,  $P < 0.05$  and  $r = 0.673$ ,  $P < 0.05$ ) (Figure 3(B,C)), whereas no significant correlations between these monoamines were present in the SCC (Figure 4(B,C)). In the AM and the DH no significant differences in the monoamine levels and their metabolites were observed between the control and scGOS:lcFOS diet groups (figure S3).

### Serotonergic system

The mRNA expression of *Htr1a* was significant decreased in the PFC ( $P < 0.01$ ) in the scGOS:lcFOS receiving mice, whereas the expression of *Htr1b* was unchanged. As the tryptophan and the 5-HT levels in the PFC were decreased in the mice receiving the scGOS:lcFOS diet, scGOS:lcFOS might influence the 5-HT synthesis from tryptophan. This is regulated by tryptophan hydroxylase 2 (TPH2). However, the mRNA expression of *Tph2* was unaffected by the dietary intervention (Figure 5(A–C)). In the SSC the mRNA expression of *Htr1a* trended towards a decrease ( $P < 0.1$ ) in the scGOS:lcFOS receiving mice, whereas the expression of *Htr1b* and *Tph2* were unchanged (Figure 5(E–G)). In the AM and the DH, the mRNA expression of the tested markers was unchanged between the dietary groups (figure S4). These data are preliminary as the sample size was small.



**Figure 2.** Anxiety-like, repetitive and social behavioural effects of scGOS:lcFOS. (A) scGOS:lcFOS receiving mice emitted significantly less ultrasonic vocalisation calls (USV). (B) Grooming frequency significantly increased over time in both control and scGOS:lcFOS receiving mice (diet:  $P = 0.08$ , age:  $P < 0.000001$ , interaction:  $P < 0.05$ ). In adulthood the grooming frequency was significantly lower in the scGOS:lcFOS receiving mice compared with the control mice. (C) The grooming duration was unaffected over time and also unaffected by scGOS:lcFOS (diet: ns, age: ns, interaction: ns). (D) The number of buried marbles was increased across age independent of the dietary supplementation (diet:  $P < 0.0001$ , age:  $P < 0.001$ , interaction:  $P > 0.05$ ). In early adulthood and adulthood the scGOS:lcFOS receiving mice buried significantly less marbles compared with control. (E) In the presence of a target mouse the scGOS:lcFOS receiving mice spent more time in the interaction zone compared with control. No differences between the groups in time the mice spent in the zone in absence of target. One scGOS:lcFOS receiving mouse in absence of target and one control mouse in presence of target were excluded as significant outliers (Grubbs test 293 s and 0 s, respectively). (F) The locomotion activity measured by distance moved was decreased in the scGOS:lcFOS receiving mice in both absence and presence of a target mouse. One scGOS:lcFOS receiving mouse in presence of target was excluded as significant outlier (Grubbs test 6200 cm). The data are shown as individually data points, colour indicating the litter, and mean  $\pm$  SEM. A-F were analysed with linear mixed models followed by Sidak's multiple comparison post-hoc test. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ , a=\*\*\* compared with adolescence within diet group, b=\*\*\*\*\* compared with adolescence within diet group, c=\* compared with adolescence within diet group, d=\*\* compared with adolescence within diet group, ns = not significant. A: control group  $n = 11$ , scGOS:lcFOS group  $n = 7$ , B-F:  $n = 9-10$  per group.

### Brain-derived neurotrophic factor (*Bdnf*)

The mRNA expression of *Bdnf* significantly increased in the PFC ( $P < 0.05$ ) (Figure 5(D)), but not in the SSC (Figure 5(H)) or AM (figure S5) of the scGOS:lcFOS mice.

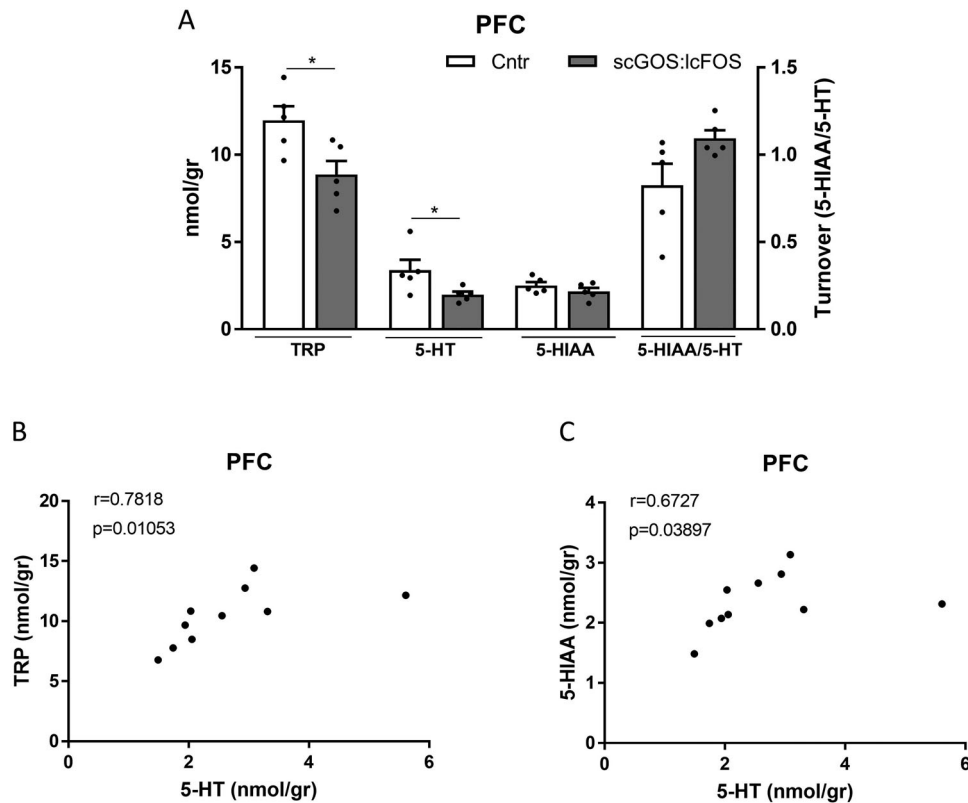
### Caecal SCFAs and branched SCFAs levels

The absolute levels of the bacterial fermentation end-products, AA ( $P < 0.05$ ), PA ( $P < 0.01$ ) and BA ( $P < 0.01$ ) were increased in the caecum content of the mice fed the scGOS:lcFOS diet (Figure 6(A-C)). In contrast, compared to the control group, the levels of protein-

derived fermentation end-products VA (not significant, Figure 6(D)), and the branched SCFAs iBA and iVA all decreased in the scGOS:lcFOS group ( $P < 0.0001$  for both, Figure 6(E,F)).

### Faecal microbiota composition

The 16S rRNA-gene sequencing of the collected faecal pellets resulted in an average sequence depth of 351,548 (SD = 220,402) reads per sample (table S3). Species richness by Chao1 index was significantly decreased in scGOS:lcFOS vs control at week 4 ( $P < 0.01$ ). The species diversity (based on Shannon index)



**Figure 3.** The PFC levels of tryptophan, 5-HT, the 5-HT metabolite 5-HIAA and the serotonin turnover (5-HIAA/5-HT). (A) The tryptophan level was significantly decreased in the scGOS:lcFOS receiving mice. The serotonin level was significantly decreased in the scGOS:lcFOS receiving mice. The 5-HIAA level and the serotonin turnover were not significantly different. The tryptophan levels and the serotonin levels (B) as well as the 5-HT and the 5-HIAA levels (C) correlated significantly. (A) Data shown as individually data points and mean  $\pm$  SEM. A: Analysed by student t-test; (B,C) analysed by spearman correlation. \*  $P < 0.05$ . A–C:  $n = 5$  per group. TRP: tryptophan, 5-HT: 5-hydroxytryptamine (serotonin), 5-HIAA: 5-hydroxyindolacetic acid.

was not significantly different between the dietary groups at the different ages (Figure 7(B)). The PRC ordination method [27] was used to assess the age dependent effects on the intestinal microbiota composition in scGOS:lcFOS fed mice compared to control fed mice. The microbial composition over time was significantly dependent on the diet (MCPT interaction,  $P = 0.001$ ). The top 10 associated bacterial genera from this PRC analysis were further evaluated by differential abundance testing, which confirmed that 9 of the 10 identified genera were differentially abundant for at least 2 repeated measurements (Figure 7(C) and table S4). In sum, an increase of 3 genera within the order of *Bacteroidales* was observed for scGOS:lcFOS compared to control, as well as an increase of the genus *Dorea* within the family of *Lachnospiraceae* and an unknown genus of the family *Erysipelotrichaceae*. In contrast, a decrease was observed for 3 unknown genera within the family of *Peptostreptococcaceae*, and the genus *Anaerovorax* within the Clostridiales family XIII (Figure 7(A,C)).

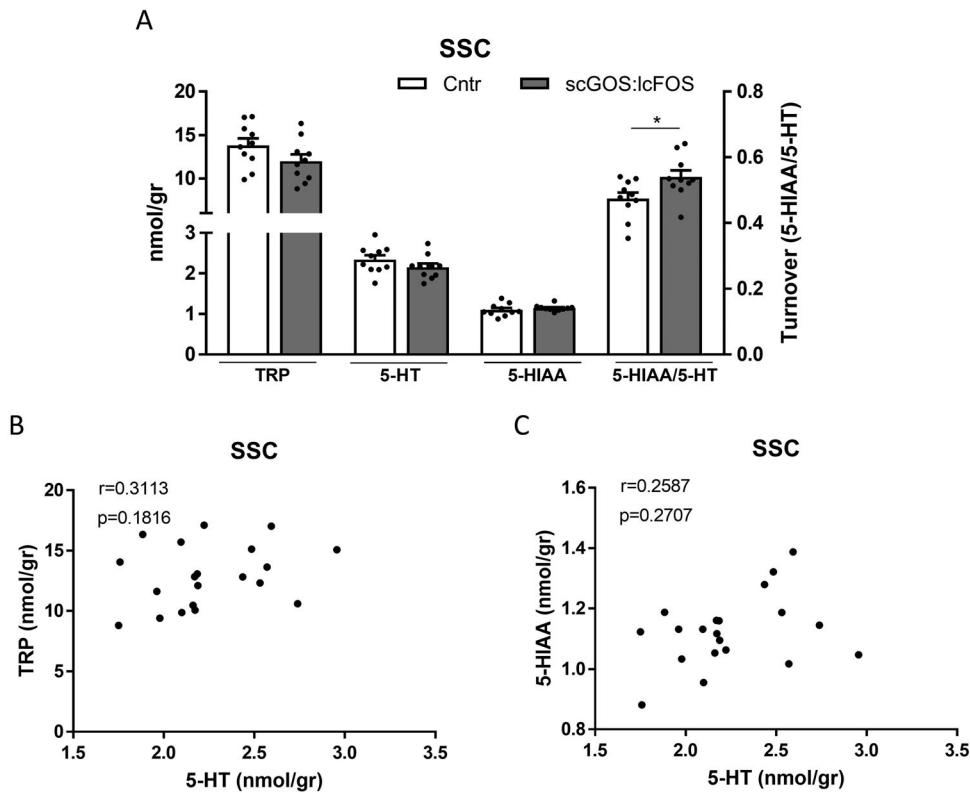
A principal component analysis (PCA) was used to evaluate the microbiota compositional differences in

adulthood between the dietary groups and to correlate these with the adulthood behavioural assessments as well as the caecal concentrations of SCFAs (Figure 7 (D)). The PCA analysis confirmed the distinct microbiota composition of the two dietary groups. The compositional differences between scGOS:lcFOS and control in adulthood correlated with increased sociability, lower number of marbles buried and lower frequency in grooming behaviour. Moreover, as measured by the caecal levels of SCFAs, the microbial composition also correlated positively with the changes in microbial activity.

## Discussion

Prebiotic fibres are known to be effective modulators of the intestinal microbiota composition and activity and may thereby positively influence health through immune- and neuromodulation [30]. However, little is known about the effect of dietary prebiotic fibres on the development of the nervous system [31]. Here, we demonstrated that dietary supplementation with scGOS:lcFOS in healthy male BALB/cByJ, started on



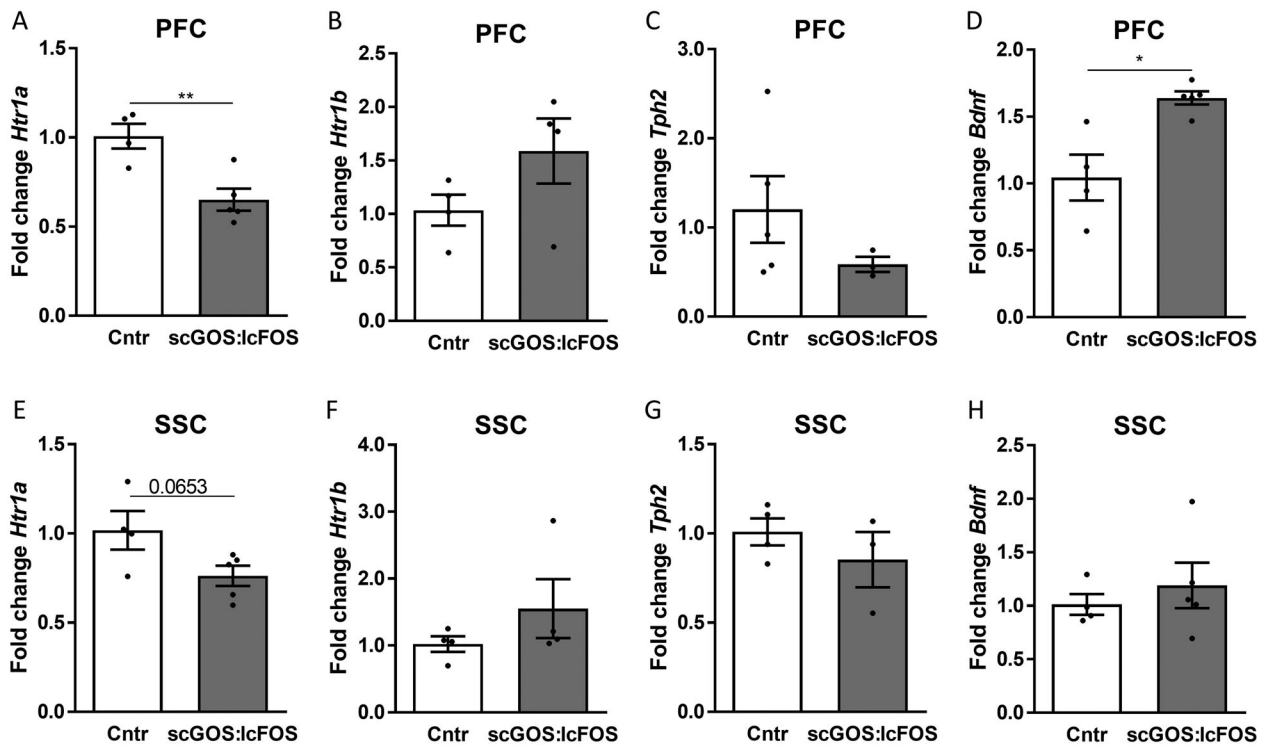


**Figure 4.** The SSC levels of tryptophan, 5-HT, the 5-HT metabolite 5-HIAA and the serotonin turnover (5-HIAA/5-HT). (A) The tryptophan, 5-HT and 5-HIAA levels were not significantly different between the scGOS:lcFOS and control diet receiving mice. The serotonin turnover was significantly increased in the scGOS:lcFOS receiving mice. The tryptophan and the 5-HT levels (B) as well as the 5-HT and the 5-HIAA levels (C) did not correlate. (A) Data shown as individually data points and mean  $\pm$  SEM. A: Analysed by student *t*-test; (B,C): Analysed by spearman correlation. \*  $P < 0.05$ . A:  $n = 10$  per group; (B, C)  $n = 10$ . TRP: tryptophan, 5-HT: 5-hydroxytryptamine (serotonin), 5-HIAA: 5-hydroxyindolacetic acid.

the day of birth, was associated with altered intestinal microbiota composition and increased saccharolytic fermentation and decreased proteolytic fermentation activity. These microbial changes were accompanied with reduced stereotypic and anxiety-like behaviour throughout life and improved social behaviour in adulthood.

The first effect of scGOS:lcFOS on behaviour in our study was already observed in early life in the ultrasonic distress vocalization test. The pups from dams fed the scGOS:lcFOS diet, emitted fewer USV calls compared to control pups. This indicates reduced anxiety-like behaviour of the pups [32] and might be a result of modulation of beneficial intestinal bacteria by scGOS:lcFOS leading to improved behaviour. On the other hand, a decreased number of USV calls by pups might indicate better maternal responsiveness [33], which may lead to improved behaviour in the pups later in life. Possibly, the maternal effects of scGOS:lcFOS on the neurodevelopment of the pups could be additive to the direct effects of scGOS:lcFOS on the brain of the pups or more relevant. To confirm this, maternal behaviour has to be measured in future studies.

During adolescence, early adulthood and adulthood, anxiety-like behaviour was reduced in mice exposed to dietary scGOS:lcFOS. This is in line with the observation that a lower number of neonatal USV calls is related to reduced anxiety-like behaviour later in life [34]. In adulthood, the reduced anxiety-like behaviour was accompanied by improved social interaction in the scGOS:lcFOS receiving mice. During the social interaction test, locomotion activity was reduced in mice fed the scGOS:lcFOS diet in presence and absence of target mouse. The reduced locomotion did not confound the time spent in the interaction zone. We used male BALB/c mice because behaviour-related brain disorders like autism spectrum disorder is more prevalent in males [35] and the BALB/c behavioural phenotype is represented by low sociability and high levels of anxiety-like behaviour [36]. Our findings that scGOS:lcFOS reduced anxiety-like behaviour and improved sociability is in agreement with a recently published study by Burokas et al. using a similar prebiotic combination [2]. In their research, the prebiotic combination GOS:FOS (1:1) given during adulthood for 10 weeks

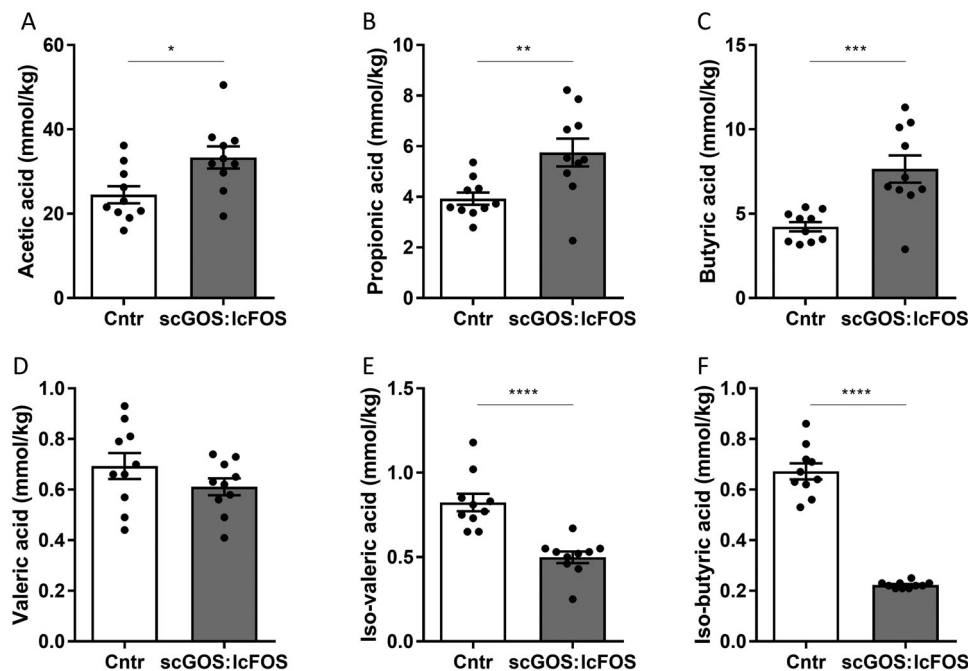


**Figure 5.** The mRNA expression of *Htr1a*, *Htr1b*, *Tph2* and *Bdnf* in the PFC and SSC. (A) In the PFC the mRNA expression of *Htr1a* was significantly decreased in the scGOS:lcFOS receiving mice. The *Htr1b* (B) and *Tph2* (C) mRNA expressions were unchanged. The mRNA expression of *Bdnf* was significantly increased in the scGOS:lcFOS receiving mice (D). In the SSC the mRNA expression of *Htr1a* trended towards a decrease in the scGOS:lcFOS group (E). The *Htr1b* (F), *Tph2* (G) and *Bdnf* (H) mRNA expression was not significantly changed between the groups. A-H: Data shown as individually data points and mean  $\pm$  SEM. A-H: Analysed by student *t*-test. \*  $P < 0.05$ , \*\*  $P < 0.01$ . (A-H)  $n = 3-5$  per group. PFC: prefrontal cortex, SSC: somatosensory cortex, *Htr1a*: serotonin receptor 1a, *Htr1b*: serotonin 1b receptor, *Tph2*: tryptophan hydroxylase 2, *Bdnf*: Brain derived neurotrophic factor.

reduced the anxiety-like behaviour in healthy mice [2]. In contrast to our study, prebiotic supplementation had no effect on social interaction, however, the GOS:FOS receiving mice showed significantly increased number of prosocial events [2]. Other prebiotic mixtures also have behavioural modulatory capacities; juvenile rats and young pigs exposed to a prebiotic mixture of polydextrose and GOS showed reduced stress-induced behaviour and improved explorative behaviour, respectively [9,10]. These behavioural changes were accompanied with increased *Lactobacillus* spp. [9]. Together these results suggest the capability of prebiotic fibres to modulate behaviour.

In addition to the behavioural changes, scGOS:lcFOS also influenced the neurochemistry of the brain in adulthood. It significantly affected the serotonergic system mainly in the PFC indicated by reduced tryptophan and 5-HT levels and lower mRNA expression of the serotonin receptor 1A, however these are preliminary data due to small sample size. In the GOS:FOS study by Burokas et al. [2], the plasma level of tryptophan was lowered after GOS:FOS administration. Together with our data this may indicate that the combination of GOS

and FOS may be able to stimulate the growth and/or function of tryptophan utilizing intestinal bacteria. Several intestinal bacteria, for example *Escherichia coli*, *Lactobacillus* and *Bacteroides* [37] possess tryptophanase, an enzyme that converts tryptophan into metabolites like indole [38]. The scGOS:lcFOS induced changes of the microbiota could lead to lower availability of tryptophan for the brain leading to lower levels of 5-HT. The decreased level of 5-HT is in contrast with the monoamine theory stating that higher levels of 5-HT and tryptophan reduce depressive and anxiety symptoms [39]. However, the monoamine theory is based on the available monoamine levels in the synaptic cleft and in this study, we measured total 5-HT levels including unreleased 5-HT in the presynaptic neurons. In other murine prebiotic studies, the total 5-HT level was either increased or unaffected in the PFC [2] or the frontal cortex [40]. However, in a comprehensive meta-study by Ruhé et al. [41] systemic depletion of tryptophan and 5-HT did not lead to decreased mood in healthy human controls. Considering the available preliminary data, the causal role of changed 5-HT and tryptophan levels on behaviour in a healthy situation is inconclusive.



**Figure 6.** Caecal SCFA. The levels of the SCFAs acetic (A), propionic (B) and butyric (C) acids were significantly increased in the scGOS:lcFOS group. (D) The level of valeric acid was unaffected by scGOS:lcFOS and the levels of the branched SCFA iso-valeric acid (E) and iso-butyric acid (F) were significantly decreased in the scGOS:lcFOS group. (A–F) Data shown as individually data points and mean  $\pm$  SEM. A–F: Analysed by student *t*-test. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ . (A–F):  $n = 10$  per group. SCFA: short-chain fatty acids.

The 5-HT<sub>1A</sub> receptor has an essential role in anxiety [42]. Activating the receptor with a 5-HT<sub>1A</sub> agonist induces anxiogenic effects which are inhibited by a 5-HT<sub>1A</sub> antagonist [43]. In our study preliminary data showed that decreased (mRNA) *Htr1a* expression in the PFC was associated with reduced anxiety-like behaviour. Due to limited brain material the mRNA levels were measured in a small sample size and therefore a limitation of this study. During adolescence, signalling of 5-HT through the 5-HT<sub>1A</sub> receptor is important and its disruption can lead to an increase in anxiety later in life [44]. The serotonin signalling during adolescence might be stimulated by scGOS:lcFOS supplementation and consequently lead to reduced anxiety-like behaviour later in life.

The neurotrophin BDNF is important in neural circuit development and in regulation of mature neural circuits [45]. Higher BDNF levels in the hippocampus, striatum and hypothalamus are associated with improved social interaction in mice [46, 47]. *Bdnf* mRNA and protein expression were enhanced in the hippocampus of adult mice and rats after prebiotic supplementation [2, 48]. Although the hippocampal (mRNA) *Bdnf* level was unavailable in our study due to insufficient material, the (mRNA) *Bdnf* level in the PFC was increased in scGOS:lcFOS fed mice. This is in line with a study in healthy rats, in which a mix of

prebiotics including GOS resulted in an increase of (mRNA) *Bdnf* in the PFC [9], indicating a beneficial effect of specific prebiotics in behaviour development.

The prebiotics modulated the intestinal microbiota in the mice and led to differences in bacterial richness and marked differences in taxonomic composition. These included increased relative abundances of the genus *Dorea* and several genera of *Bacteroidales* and *Erysipelotrichales*, and decreased relative abundances of the genus *Anaerovorax* and several genera of *Peptostreptococcaceae* (all within the order of *Clostridiales*). The prebiotic effects observed in these mice differ markedly from observations in human infants, in which prebiotics predominantly leads to increased levels of bifidobacteria [11]. Although the mice receiving scGOS:lcFOS showed enhanced levels of bifidobacteria compared to control, the differences were not significant (figure S5). The modulatory effects of scGOS:lcFOS likely depends on the already resident mouse microbiota. This is supported by the findings in another mouse study, in which only the combination of scGOS:lcFOS with the probiotic *Bifidobacterium breve* M-16 V showed a bifidogenic effect in contrast to the intervention containing the prebiotic components only [49]. Possibly, the mice specific bifidobacterial species are inefficient in utilizing scGOS:lcFOS or are outcompeted by other bacterial species. Indeed, we observed that the supplementation with



scGOS:lcFOS mainly led to increased relative abundances of the more dominant *Bacteroidales* spp., which are known to degrade similar complex carbohydrates as used in this experiment [50, 51]. Similarly to our study, Burokas et al. found increased levels of the *Bacteroidales* S24-7 group, which is a prominent but yet uncultured group in the murine gut that may have the machinery to degrade scGOS:lcFOS [52]. However, the other bacterial groups reported to respond to the scGOS:lcFOS intervention were distinct from our observations. This could be due to several factors such as the different genetic background of the mice (BALB/c vs C57BL/6J), supplier, housing (group vs individual), diets and ratio of used prebiotic mixture, and location of the laboratory. Moreover, the selective effects of the prebiotics on the *Bacteroidales* spp. may have stimulated the putative butyrate-producing microbial groups of *Erysipelotrichales* [53] through cross-feeding mechanisms [4]. Therefore, investigating the composition of intestinal microbiota as a whole in relation to in depth metabolomics is important to obtain functional information on precisely how the intestinal microbiota is influenced by scGOS:lcFOS and influences brain and behaviour.

In contrast to the distinct bacterial composition between our and the Burokas study, the bacterial activity assessed by caecal SCFAs were similar. The acetate and propionate levels were increased, and the iso-butyrate level was decreased, and this was also observed by Burokas et al. [2]. Additionally, in our study, the butyrate level was increased and the iso-valerate level was decreased. Overall, the decrease of branched SCFAs (iso-butyrate and iso-valerate), which are fermentation products from degradation of proteins and amino acids [54, 55], indicates that the scGOS:lcFOS diet shifted the intestinal microbiota from a more proteolytic profile to a more saccharolytic profile. The latter is typically associated with higher levels of acetate, propionate and butyrate, and metabolite profiles considered to be more beneficial for colonic health [56].

Changes in intestinal microbiota can affect brain and behaviour through the gut-brain axis through several mechanisms, including microbe-derived molecules like SCFAs, and tryptophan metabolites neuroactive molecules like neurotransmitters and neuronal signalling by stimulation of the vagus nerve [57]. As SCFAs are fermentation products from scGOS:lcFOS and SCFAs have been shown to reduce anxiety-like and depressive-like behaviour in mice [58], these mediators might play a role in the behavioural changes observed in our study. How this communication between the SCFAs and the brain occurs is unknown, however, signalling through the vagus nerve [10] or improved integrity of the blood

brain barrier could play a role [59]. In the central nervous system, SCFAs (among others) play a role in the homeostasis of microglia cells, macrophage-like cells in the brain. Studies in germ-free mice indicated that microglia cells are affected by the intestinal microbiota [60] indicating that microglial function might be influenced by scGOS:lcFOS in an immunomodulatory way.

In conclusion, scGOS:lcFOS from the day of birth modulates the composition and activity of the intestinal microbiota, and is associated with reduced anxiety-like and improved social behaviour in healthy male BALB/c mice. To evaluate the exact effects in early life, it is essential to investigate brain neurochemistry at several time points throughout phases of development. Here, we provide further evidence of the potential beneficial effects of scGOS:lcFOS on behaviour at several developmental stages throughout life and strengthen the insights in the interplay between the developing intestine and brain.

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## Disclosure statement

Johan Garssen, Leon Knippels, Kees van Limpt, Harm Wopereis and Jan Knol are employees of Danone Nutricia Research. Cindy de Waard is owner of CAM practice specialised in gut health.

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**Harm Wopereis** obtained his PhD in microbiology in 2019. In 2006 he joined the Gut Biology and Microbiology department

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**Cindy de Waard** obtained a BSc in Biomedical Sciences and a MSc in Pharmaceutical Sciences. Owner of a Complementary and Additive Medicine (CAM) practice specialised in gut health.

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**Johan Garssen** studied medicine and biology at the Free University, Amsterdam, Netherlands. He specialized in immunology and pharmacology and finished both studies in 1987 cum laude. He finished his PhD thesis at the University of Utrecht in 1991 on the role of T cells in respiratory allergy. This PhD program and a postdoc period was partly performed at Yale University, New Haven, USA. After the postdoc period

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**Aletta D. Kraneveld** (MSc Pharmacy & pharmacologist) was appointed as full professor Interdisciplinary Translational Pharmacology at the Faculty of Science and the Faculty of Veterinary Medicine of the Utrecht University in 2016. She has published >115 papers (H-index: 39). Besides science leading the neuroimmunopharmacology group, she is an active member of several boards of (inter)national scientific and societal organizations (Dutch Society of Pharmacology, EPHAR, IUPHAR, Netherlands Federation of Innovative Drug research; Diversity committee UU; Food Lives-NL). Kraneveld’s current research interests involve targeting the interaction between innate and adaptive immunity as well as host-microbiome interactions in chronic (inflammatory) diseases with pharmaceutical as well as nutritional interventions. The Kraneveld group is focusing to in depth study the role of the gut-immune-brain-axis in neurodevelopmental and neurodegenerative disorders. Results will further enhance the knowledge of the interaction of microbiome with the immune and nervous systems in chronic conditions in the gut and CNS. Aletta Kraneveld has set up a program that is a (inter)national neuro-immune platform where academia, patient organisations and industry meet for research on the gut-immune-brain axis as target for medicine and medical food concepts.

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