Doctoral School of Basic Medical Sciences

The impact of modifying the gastrointestinal microbiome on social behavior and fatty acid composition

Doctoral (PhD) Thesis

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I. Introduction

In recent years, our knowledge of the intestinal microbiota has greatly improved. Dysfunctions of the intestinal microbiome are being encountered more frequently, both in connection with certain diseases affecting the central nervous system (major depressive states, autism spectrum disorder, Alzheimer's disease, etc.) and due to its increasing impact on social health care. These diseases impact a broader range of social strata and age groups, and their general characteristic is that their treatment is predominantly symptomatic rather than causal. Our research is predicated on the hypothesis that the gastrointestinal (GI) microbiome not only plays a significant role in regulating peripheral functions but also have a profound impact on the central nervous system functions, as a reason of that it can also influencing the behavior. The composition and balance of the intestinal microbiome i.e. the community of commensal, symbiotic and pathogenic microorganisms established in the gastrointestinal tract, have a significant effect on the physiological functions of the host. This is further supported by the fact that the disruption of the balance of this complex system leads to changes both peripherally and centrally. Considering the bidirectional connection and our current knowledge, it is hypothesized that dysbiosis of the gastrointestinal microbiome and certain disorders affecting the central nervous system are highly related.

In autism spectrum disorder (ASD), alterations in the microbiome profile can be observed, and differences in the substances produced by microbes are also detectable. The relationship between the autism spectrum disorder and the GI microbiome is supported by various pieces of evidences. Consequently, several hypotheses have been proposed to elucidate the potential mechanism linking ASD with the gut microbiome community. These hypotheses also aim to support the idea that one possible origin of the disorder could be in the disturbed microbiome.

As a result of various treatments, we are able to induce significant changes in the composition of the intestinal microbiome even in adulthood, which can play a crucial role in the therapeutic approach to several diseases. We are able to influence intestinal microbiota through various techniques, including dietary modifications, probiotics (living microorganisms that have a beneficial effect on the host), prebiotics (indigestible food ingredients that selectively stimulate the growth and activity of certain beneficial bacteria), symbiotics (combination of probiotics and prebiotics), postbiotics (metabolism products or components of beneficial microorganisms), antibiotics and Fecal microbiota transplantation.

Fatty acids are important constituents of all multicellular organisms, primarily as components of the phospholipid bilayers of the cell membranes, and they also have an essential

role in normal growth and development. They also play a substantial role in shaping the composition of the GI microbiome, as a result, qualitative and quantitative modifications in fatty acids have a significant impact on the complex ecological system of the intestinal microbiome. However, it is already known that the relationship between fatty acids and the GI microbiota is not only unidirectional. Numerous research confirms that alterations in the whole microbiome community can also affect the composition and the quantity of fatty acids. The main focus of these studies is to explore the connection between the microbiome and the short-chain fatty acids, as these acids (acetic acid, propionic acid and butyric acid) are the main end products produced during the bacterial fermentation of complex carbohydrates. The consequences of alterations in the microbiome can lead to modifications not only in the composition of short-chain fatty acids but also in the content of longer-chain fatty acids. It has been confirmed that the intestinal microbiome plays a crucial role in the production of certain fatty acids. Consequently, the relationship between the intestinal microbiome community and the fatty acids present in the host may be more significant than previously thought. Not only nutrition, but also the structure of the gut microbiome appears to play an important role in determining the quality and the quantity of fatty acids.

II. Objectives

1. The first objective of our studies was to assess and elucidate the effects of qualitative and quantitative disturbances in the gut microbiome on social behavioral responses, thereby enhancing our understanding of the various roles that intestinal bacteria play in behavior.

2. The secondary goal of our studies was to unravel the effects of qualitative and quantitative disturbances in the intestinal microbiome on the detailed fatty acid spectrum, thereby to better understand of the role of intestinal bacteria in fatty acid production.

- Our aim was to investigate whether chronic broad-spectrum antibiotic treatment could induce ASD-like behavioral symptoms in social behavior during adulthood, furthermore, these behavioral changes could be compared with the alterations observed in the valproic acid-induced ASD animal model.

- Our main objective regarding our probiotic mixture was to determinate whether this treatment could reverse the behavioral changes induced by antibiotics.

- Our further goal was to assess whether treatment with the probiotic mixture could alleviate and reduce the social behavioral alterations observed in the valproic acid-induced ASD model.

- Our another aim was to explore the effects of chronic broad-spectrum antibiotic treatment on body weight, food and water intake.

- We also aimed to clarify whether modifications in intestinal bacteria resulting from antibiotics treatment could influence the concentration and weight percentage ratio of fatty acids.

- Additionally, we intended to assess whether the probiotic mixture could alter the fatty acid composition.

- Finally, our objective was to examine whether the probiotic mixture could reverse the changes in the composition of fatty acids induced by antibiotics.

III. Materials and methods

III.1. Subjects

In the present study, in total, 72 male Wistar laboratory rats were used. The treatments started when the animals reached 10 weeks of age. The animals were kept individually in a light and temperature-controlled room. All experimental groups received the same laboratory food pellets and tap water ad libitum. The animals received daily handling in order to acclimate them to human presence and touch. All animal experiments were conducted according to federal and local ethical guidelines, and the protocols were approved by the National Scientific Ethical Committee on Animal Experimentation of Hungary (BA02/2000–15/2020 and BA02/2000–16/2020, Pécs University, Medical School; Hungarian Government Decree, 40/2013. (II. 14.); NIH Guidelines, 1997; European Community Council Directive 86/609/EEC 1986, 2006; European Directive 2010/63/EU of the European Parliament). The present study is reported in accordance with ARRIVE guidelines.

III.2. Treatments

III.2.1. Treatments with antibiotics and probiotic

Animals have been divided randomly into four groups: 1. Control group (control); 2. Antibiotics treated group (ABx); 3. Antibiotics and probiotic treated group (ABx+probiotic); 4. Probiotic treated group (probiotic) (Figure1). The antibiotics treated groups received broad-spectrum antibiotics mixture for 4 weeks. The antibiotics cocktail was dissolved in their drinking water to avoid any chronic stress-induced adverse effects. The antibiotics mixture consisted of 5 antibiotics mixture (ampicillin 1 g/l, vancomycin 500 mg/l, ciprofloxacin HCl 20 mg/l, imipenem 250 mg/l and metronidazole 1 g/l). This antibiotics cocktail was replaced by freshly made cocktail every 3 days. After this antibiotics exposure in the ABx+probiotic group, and the probiotic group were given our probiotic mixture (know-how under the license of the University of Pécs (422.lbh.5.(2019.09.05)) contained four beneficial bacterial species (*Lactobacillus spp., Bifidobacterium spp.*) of specified cfu/d (colony forming units/day), oral gavage every day for 2 weeks. The origin of the strains was Leibnitz, institut DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Deutschland. *Lactobacillus spp.* strains were cultivated in 100–100 mL liquid Rogosa medium (OXOID Ltd. UK). The *Bifidobacterium spp*. were cultivated on fastidious anaerobic agar CE plates and broth (Neogen

Europe Ltd. UK). Anaerobic conditions were produced in anaerobic jar with GEnbag anaer (BioMérieux SA France). During the two-week treatment period, the mixture was produced daily and used immediately.

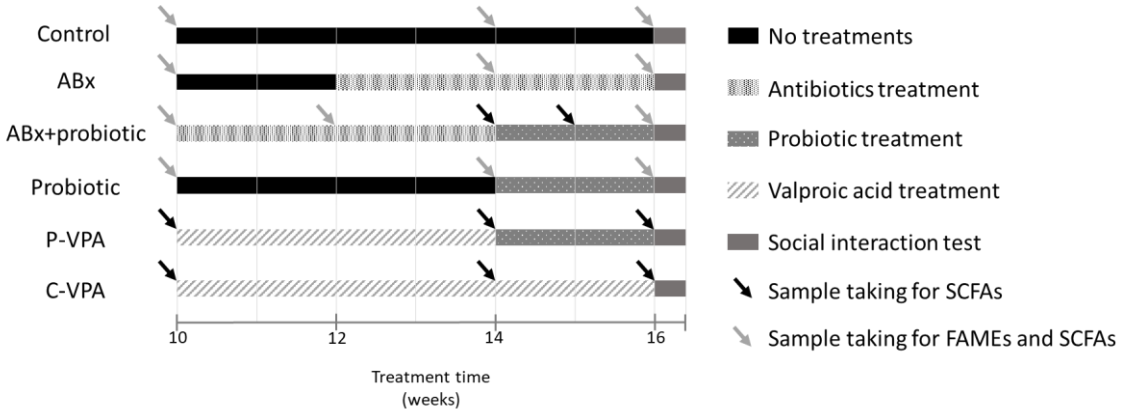

Figure 1 *Experimental arrangement of the treatments. Control: control group, ABx: broad-spectrum antibiotics treated group, ABx+probiotic: broad-spectrum antibiotics and probiotic treated group, Probiotic: probiotic treated group, P-VPA: valproic acid and probiotic treated group, C-VPA: valproic acid treated group. The arrows show the stool sampling times, the black arrows indicate the sampling times only for the analysis of short-chain fatty acids (SCFAs), the grey arrows indicate the sampling times for the analysis of short-chain fatty acids (SCFAs) and fatty acid methyl esters (FAMEs) at the same time.*

III.2.2. Treatment with lithium chloride

To definitely exclude the possibility that the observed behavioral differences were not the consequence of the intestinal discomfort due to the antibiotics treatment, we conducted a pilot experiment. Lithium chloride (LiCl) is a well-known toxicous chemical which causes visceral illness and in this pilot experiment it was used to generate intestinal discomfort. 8 rats were injected intraperitoneally with LiCl (125 mg/kg/ml) and half hour after the injection three chamber social interaction test was conducted.

III.2.3. Treatment with valproic acid

Valproic acid-induced ASD animal model was used to imitate autism spectrum disorder. Male and female Wistar rats were mated one overnight, and then after successful fertilization pregnant female rats received a single intraperitoneal injection of 500 mg/bwkg valproic acid

(VPA) (Sigma-Aldrich; P4543) dissolved in physiological saline at a concentration of 250 mg/ml on the 12.5th day of gestation, and control females were injected with physiological saline at the same time. Females were housed individually and were allowed to raise their own litters. Male offsprings, when reached 8 weeks, were separated and randomly divided into two groups: probiotic treated valproate group (P-VPA); 2. control valproate group (C-VPA) (Figure 1). The P-VPA group were given the above described probiotic mixture every day for 2 weeks.

III.3. Experiments

III.3.1. Body weight, food and water intake measurements

Body weight, food and water intake measurements were performed after the animals reached 10 weeks of age. The body weight of the animals was measured every 3 days, and their food and water intake was monitored daily throughout the experiments.

III.3.2. Three chambered social interaction test

A three chambered social interaction task was used to assess social behavior. The apparatus was divided into three chambers: the nonsocial zone (60 × 40 cm), the social zone (60 × 40 cm) and the centre (30 × 40 cm). The non-social and the social zone contained small circular wire cages with a diameter of 18 cm. Before the sociability task experimental rats immediately were given a habituation session. They were placed into the centre of the apparatus, where they were allowed to explore it for 10 min. Right away this habituation session test animal was placed in their home cage for 3 min, while a stranger rat of the same strain and sex was placed into one of the two rat cages in the side chambers. Therefore, one of the side chambers which contained a stranger rat would be the social zone and the other chamber, where the cage remained empty would be the non-social zone. Following that test animals were provided 10 min to freely explore the whole apparatus. The entire experimental period was recorded and analyzed by recorded camera shots processed by Noldus EthoVison System (Noldus Information Technology, The Netherlands). We measured the total distance moved, time spent in the side chambers, and during the sociability task latencies to first entry to the side chambers were also determined. Furthermore, direct interactions with either the stranger rat- or empty cages were counted. All types of exploratory behavior were noted, and the sociability index (time spent in the social zone − time spent in the non-social zone)/(time spent in the social zone + time spent

in the non-social zone) was also used to indicate a preference to interact with or avoid the stranger rat.

III.4. Examinations of chromatography

Fresh fecal pellets were collected in sterile Eppendorf tubes under controlled conditions during the treatments and stored in −80 ℃ until use. These samples were collected at 3-5 time points for testing short-chain fatty acids and at 3 time points (before, during and after treatments) for measuring fatty acid methyl esters.

III.4.1. Short-chain fatty acids (SCFAs) analysis

100 mg of faecal samples were weighted out and vortex-mixed with 1 ml of distilled water. After standing for 10 min at room temperature, 100 µl (2 mmol/l) Heptanoic acid (Sigma-Aldrich; 43858) as an internal standard was added to them and the samples were centrifuged at 10,000 rpm for 5 min. Then supernatant fluids were collected and filtered before being transferred to the vials. Standard solutions of acetic acid (Sigma-Aldrich; A6283), propionic acid (Sigma-Aldrich; 94425) and butyric acid (Sigma-Aldrich; 19215) were used for calibration. The concentration of SCFAs analyses were carried out on an Agilent 6890 N gas chromatograph with a 5975 mass spectrometer detector (Agilent, Santa Clara, CA, USA) fitted with a HP-INNOWAX column (30 m × 0.25 mm × 0.25 µm; Agilent). Helium was used as the carrier gas (1.5 ml/min). The initial oven temperature was 80 ℃ held for 1 min and ramped up to 200 °C at 20 °C/min and held for 2 min. Split injection mode was used (250 °C), the injection ratio was 20:1. The injection volume was 1 µl.

III.4.2. Fatty acid methyl esters (FAMEs) analysis

From the collected samples (control, ABx, ABx+probiotic, probiotic groups, before (1st sample), during (2nd sample) and at the end of the treatments (3rd sample)) approximately 100 mg fecal content was placed in test tubes. We added 40 µl solution containing 60 mg methyl tridecanoate (C13:0) in 20 ml of n-hexane to each tube with the sample. We added 3 ml of methanol:hexane 4:1 (V/V) and 1–2 mg pyrogallol to the samples, then these samples were frozen for half an hour. 300 µl of acetyl chloride was slowly added to the frozen samples. The tubes were subjected to methanolysis at 100 °C for 1 h. After the tubes cooled down, 5 ml of 6% K_2CO_3 solution was slowly added to stop the reaction and to neutralize the mixture. The

tubes were vortex-mixed and centrifuged. The supernatant hexane layer was then transferred to a 2 ml screw-top vial with a micropipette. After this step, hexane was added until reaching the liquid level of 1 ml. The fatty acids analyses were carried out on a PerkinElmer Clarus 690 gas chromatograph with a flame ionization detector (PerkinElmer, USA) fitted with a Rt-2560 capillary column (100 m × 0.25 mm i.d. × 0.20 μm film thickness, Restek). Helium was used as the carrier gas (1.3 ml/min). Split injection mode was used (225 °C), the injection ratio was 20:1. The injection volume was 2 μl. The detector temperature was 300 °C. The initial oven temperature was 100 °C held for 4 min and ramped up to 250 °C at 3 °C/min and held for 25 min. The fatty acid methyl esters were analyzed based on the area under the curve calculated using TotalChrom software by PerkinElmer. Fatty acid identification was based on the retention times of external standards (PUFA3 (Supelco, St. Louis, MO, USA); GLC-674, -642, -643, -569b, -481, and -473 (Nu-Check-Prep, Elysian, MN, USA); C16:1n-9-ME (Larodan AB, Solna, Sweden) and The Bacterial Acid Methyl Esters CP Mixture (Matreya LLC., State College, PA, USA). The individual FA response factors determined from these weighed standards and the percentage area under the curve (relative concentration; w/w%) were used to calculate the weight percentage of each determined. The concentrations of fatty acids were expressed in μg /100 mg wet feces).

III.5. Histology

In order to determine the thickness of the hippocampus layers, we performed histological examinations. At the end of experiments animals were euthanized and transcardially perfused with physiological saline followed by 4% formalin solution. The brains of 6 animals/groups were fixed in 4% formalin, afterwards hippocampal areas were cut into 40 μm sections and stained using Cresyl Violet staining. The open-source image-processing software package ImageJ (NIH) was used for image analysis. The diameter of the hippocampal regions (subiculum, CA1, CA2, CA3, dentate gyrus) of the selected section (bregma −3.6 to 3.8 mm) were measured.

III.6. Statistics

In the analysis of the three chamber social interaction test, short-chain fatty acids and histological examinations were conducted using the statistical software package (IBM SPSS Statistics 22) and the significance was denoted with selection of a p value of < 0.05. In the

social interaction test the stranger- and empty cage latency, interactions with the stranger rat and the social index, further the analysis of the SCFAs and the determination of histological measurements non-parametric Kruskal–Wallis test, Mann–Whitney U-test and Friedman test were used. For the other comparisons in the three chamber social interaction test, body weight, food- and water consumptions parametric one-way ANOVA was used and Post-hoc group mean comparisons were conducted using Tukey's post hoc test.

For classifying the FAMEs sample's chromatograms Principal Component Analysis (PCA) was applied. The input data for the PCA was the area under the curve of the components. The PCA model was fitted using Eigen value decomposition method. To detect the effect of the treatments in time on the fatty acids composition and concentration we applied a nested linear mixed random effect model, where the identifiers of the rats were used as a random factor and the nested effect was the litter. In all models we used the time point of the sample taking, treatments and their interaction as explanatory factors and the models were adjusted to the baseline values. All analyses were done with R statistical software (version 4.2.1; packages: lme4, lmerTest; R Core Team, Vienna, Austria). A result was set as significant if the p value was under 0.05.

IV. Results

IV.1. Results of body weight, food and water intake

The results did not show significant differences among the examined animal groups, indicating that the treatments did not significantly influence the body weight, food and water consumption.

IV.2. Results of social interaction test

During the data analysis, the total time spent exploring the stranger rat or empty cage showed significant differences in the ABx and C-VPA treated animals compared to the other groups (Figure 2). Both treated animal groups spent significantly less time in the social zone (C-VPA: $p = 0.013$, ABx: $p = 0.006$) and more time in the non-social zone (C-VPA: $p = 0.046$, ABx: $p = 0.005$). The sociability index reduced by approximately 70% in the C-VPA and ABx group compared to the other groups ($p = 0.022$). Measurement of interactions with stranger animal and empty cage were revealed that significantly less interactions were observed with the stranger rat in the ABx ($p = 0.002$) and C-VPA animals ($p = 0.050$), moreover, ABx rats differed from the ABx + probiotic ($p = 0.025$), probiotic ($p = 0.003$) and P-VPA ($p = 0.010$) rats, too. However, we did not find any difference among the groups in the empty cage interaction.

The examination of total distance travelled and stereotype behaviors did not show significant differences among the groups.

Following treatment with lithium chloride, no significant differences were observed in the total time spent exploring the stranger rat or in the analysis of the sociability index compared to the six investigated groups.

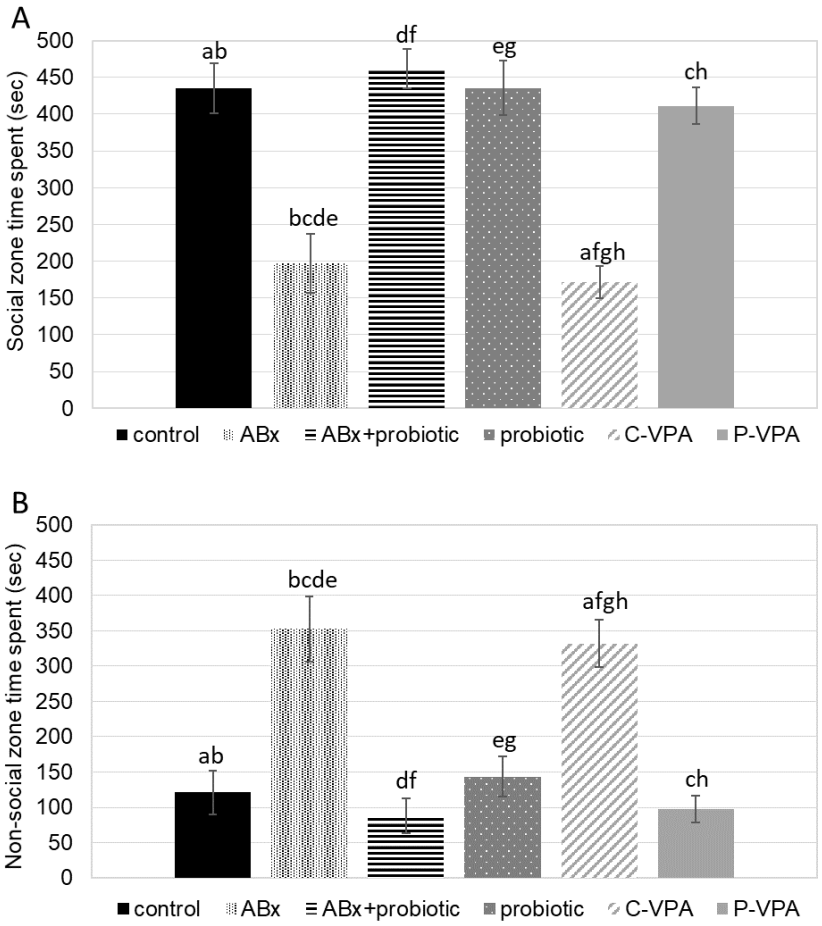

Figure 2 *Time spent with the social- (A) and non-social (B) zone exploration (s) in the three chamber social interaction test between the different groups. Control: control group; ABx: broad-spectrum antibiotics treated group; ABx+probiotic: broad-spectrum antibiotics and probiotic treated group; probiotic: probiotic treated group; C-VPA: valproic acid treated group; P-VPA: valproic acid and probiotic treated group. Data graphed as mean ± SEM (n=10/group). Significant change between groups is indicated by the same lowercase letters above the columns (a-h) (One-way ANOVA, $p < 0.05$).*

IV.3. Results of short-chain fatty acids

Following the analysis of SCFAs, a significant decrease in concentrations was observed in all three measured fatty acids after the antibiotics treatment compared to the other groups; C-VPA ($p = 0.001$), P-VPA ($p = 0.042$), ABx + probiotic ($p = 0.013$), probiotic ($p = 0.002$) and control ($p = 0.025$) groups. Additionally, acetic acid ($p = 0.001$), butyric acid ($p = 0.001$) and propionic acid ($p = 0.001$) concentrations were also diminished within the ABx group between

before and after the treatment. Similarly, in the ABx+probiotic treated group, a significant decrease ($p = 0.001$) was identified following the usage of antibiotics. It is worth noting that the probiotic treatment was able to restore this decrease to the original
state, but there were no differences before and after both of the treatments in the SCFAs concentrations. Examination of the prenatal VPA exposed rats did not present significant differences among and within these animal groups.

IV.4. Results of fatty acid methyl esters

IV.4.1. Characteristics of the chromatogram

A principal component analysis was applied to confirm the different characteristics of the chromatograms. This analysis showed that the areas under the chromatogram curve of fatty acids differed because of the different treatments. The two main principal components (PC1 and PC2) explain 80.1% of the variances of the differences in the chromatograms in the case of the second sampling time. The PCA model clearly separates the samples into two groups. The first group consists of the ABx and the ABx+probiotic; whereas, the control and the probiotic groups are in the second. Although PC1 and PC2 explain only 67.9% of the variances of the differences between the chromatograms in the case of the third sampling time, we can see that the PCA scores of the ABx+probiotic group are closer to those of the control and probiotic groups than those of the ABx group. Whereas the ABx group remains separate from the other groups (Figure 3).

IV.4.2. Results of mixed model analysis

A nested linear mixed random effect model was used to investigate the differences between the concentrations and the weight percentage ratios of the fatty acids components. The results showed that the main categories of the examined fatty acids exhibited substantial differences in the parameters we compared (time points of sample taking, treatments and their interactions) both in the analysis of the concentration and weight percentage ratio for total fatty acid content. In addition, there were significant differences between the compared parameters for most each

examined fatty acid concentrations tested, indicating that the gut microbiota affected the values of these fatty acids.

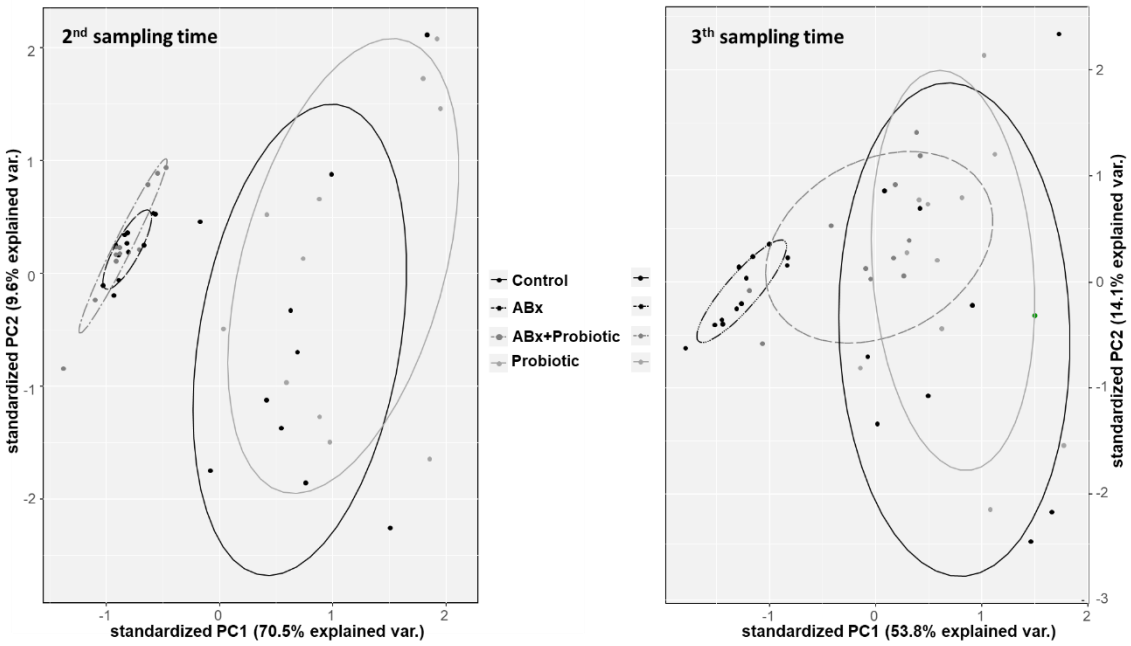

Figure 3 *Plots of the two main components (PC1 and PC2) of the principal component analyses. The graphs represent the second and third sampling time, which were made based on the areas under the chromatogram curve data. The colors illustrate the 4 groups (control, antibiotic treated (ABx), antibiotics and probiotic treated (ABx+probiotic) and probiotic treated group) and the dots represents the rat samples.*

IV.4.2.1. Saturated fatty acids (SAT)

During the analysis of saturated fatty acids, a considerable difference was demonstrated between the time points, the treatments and their interaction. This indicates that both ABx and subsequent probiotic treatment influenced the concentration and weight percentage ratio of this category, suggesting, that gastrointestinal microbiome played a major role in the production of most of the fatty acids in this category.

IV.4.2.1.1. Short saturated fatty acids (shortSAT)

The short saturated fatty acids is a subcategory of SAT, including five FAs (C4:0, C5:0, C6:0, C7:0, C8:0) in this study. This subcategory also presented significant differences in time

points, treatments and their interaction, indicating that both FA concentrations and weight percent ratios were modified by the different treatments.

IV.4.2.2. Trans and branched fatty acids

This main category consists of three coelutions (tC17:1n-7 + C18i, tC16:1n-7 + C17i and tC18-1mix) and five branched-chain saturated fatty acids, which showed large differences. There were significant differences in both concentration and weight percent values between time points, treatments and their interactions, so both the ABx and probiotic treatment effectively altered them.

IV.4.2.2.1. Branched saturated fatty acids

In this subcategory, all the examined branched SATs (C13ai, C14i, C15i, C15ai and C16i) showed strong significant differences between sampling times, treatments and their interactions in both concentration and weight percentage ratio for total fatty acid

IV.4.2.2.2. Trans fatty acids (TFAs)

During the examination of this subcategory, we obtained similar results to those observed in the analysis of its main category. The ABx treatment significantly decreased both the concentration and the weight percentage ratio of tC18:1 fatty acid, but subsequent probiotic administration was able to increase these levels to the level before the treatments.

IV.4.2.3. Cis monounsaturated fatty acids (cMUFAs)

In the cMUFAs almost all the parameters tested showed significant differences in both absolute concentration and weight percent ratio. In this category we examined 8 cMUFAs (C13:1n-1, C14:1n-5, C16:1n-9, C16:1n-7, C18:1n-9, C18:1n-7, C20:1n-9 and C22:1n-9).
Except for C22:1n-9, which showed only tendencies, most of these cMUFAs revealed significant differences in concentration values between time points, treatments, and their interactions.

IV.4.2.4. Polyunsaturated fatty acids (PUFAs)

Significant differences were observed in both concentration and weight percent ratio of PUFAs. The PUFAs category were separated in two subcategories which showed really similar differences: n-3 PUFAs (α-linolenic acid (C18:3n-3, ALA) and C20:5n-3) and n-6 PUFAs (C18:2n-6, C20:2n-6, C20:3n-6, C20:4n-6, C22:4n-6 and C22:5n-6). A further subcategory within PUFAs is the long chain polyunsaturated fatty acids (LCPUFAs) that also can be divided in 2 subgroups: n-6 LCPUFAs (C20:2n-6, C20:3n-6, C20:4n-6, C22:4n-6 and C22:5n-6) and n-3 LCPUFAs (C20:5n-3). The LCPUFAs subcategory and the n-6 LCPUFAs subgroup showed similar results as the PUFAs category in both concentration and weight percent ratios, but there were no significant differences in the n-3 LCPUFAs subgroup.

IV.4.2.5. Fatty acid composition of the pellets

We analyzed the fatty acid composition of the rodent chow and compared it with the treatment effects. The analysis revealed that out of the examined fatty acids the MUFAs were present in the highest quantity in the rat feed, alongside PUFAs and SATs. No Branched SATs were found in the animal feed. Furthermore, within the category of TFAs, tC18:1 fatty acid were present, but in a small amount. The obtained results indicate that the FAs present in the rat feces and their changes resulting from the treatments were not influenced by the FA composition of the feed.

IV.5. Results of histology

At the end of behavioral experiments, the diameter of the major hippocampal regions (subiculum, CA1, CA2, CA3, dentate gyrus) was compared among 6–6 animals/groups. Based on the results, diameter of the various hippocampal regions demonstrated extreme differences, nevertheless, the thickness of each region also revealed remarkable alterations within the groups. Therefore, our histological data, at their present form, proved not to be correctly interpretable.

V. Discussion

V.1. The effects of gastrointestinal microbiome modifications on social behavioral processes

One of the fundamental findings of the current study highlights that the antibiotics induced bacterial depletion in adulthood can elicit the same type deficits of the social interaction as those observed in the VPA autism rat model. The sociability index results, indicating a preference to the stranger rat, presented that rats of the C-VPA- and ABx groups display social interaction deficits. These findings suggest that chronic broad-spectrum antibiotics treatment in adulthood negatively affects the social behavior, moreover, it seems as if these deficits were the same type as those we could identify in the VPA rat model of ASD. This finding appears to support the notion that the antibiotics-modified microbiome can act as a causal agent and a risk factor in the development of ASD. Moreover, the pilot experiment with LiCl induced visceral illness was not able to result in reduced sociability what we detected in the microbiome depleted ABx rats. These results suggest that impaired sociability is likely cannot be due to the general consequence of visceral discomfort or pain in these animals. The above data underline the importance of disruption of the healthy balance of microbial community and its specific impact on the microbiome–gut–brain axis that leads to the deficits of social behavior regardless of whether visceral discomfort exists or not.

In our present study, it is also demonstrated that specified probiotic mixture can be a potential novel approach to improve social behavioral alterations both in the VPA- or antibiotics induced animal model. The applied probiotic mixture was able to improve the preference to the stranger rat in both of the C-VPA and ABx treated groups, thus antisocial behavior was reduced. Additionally, the present results demonstrated that the same behavior observed in the P-VPA and the ABx+probiotic groups was also seen in the control group. This indicates that the probiotic mixture not only alleviated the antisocial behavior, but also successfully restored the normal social behavior.

Despite the fact that the chronic broad spectrum antibiotics treatment and the VPA treatment developed by different mechanisms, quite similar social behavioral abnormalities were noticed, additionally, the applied probiotic mixture was able to reconstruct these behavioral phenomena just as they appear in the control rats. Regarding these consequences, it is suggested that in both models the protective effects of the probiotic treatment get exerted in the same way.

V.2. The effects of gastrointestinal microbiome modifications on short-chain fatty acids production

We hypothesized that the protective effects of the probiotic treatment get exerted similarly in both models, and the alterations in SCFAs would consequently occur as a result, since series of studies have described that SCFAs improve the gut health, regulate immune mechanisms and they may possess neuroactive properties. Despite these considerations, the present outcomes suggest that the change of concentration of the main SCFAs is not the common pathway responsible for the effects of the probiotic treatment. The analysis of the main SCFAs did not show significant differences between the VPA treated and the control animals, and, after the probiotic therapy, there were also no remarkable effects seen in the SCFAs productions.

Nevertheless, it is clear that the antibiotics treatment itself significantly decreases the concentration levels of all the examined SCFAs, referring to the highly decreased total amount of the microbiome in these animals. In spite of the fact that the probiotic mixture considerably elevated the concentration of the SCFAs after the antibiotics treatment, we did not reveal extreme alterations among the groups after the end of the treatments. However, it is reasonable to suppose that the impact of the probiotic treatment would be necessarily stronger after the antibiotics administration than in case of challenging the compact, untreated microbiome community. The present results undeniably indicate that changes in the concentrations of the 3 main SCFAs cannot be the sole causal factor determining how the probiotic mixture exerts its positive impact on the social behavior. Therefore, these observations encourage us to maintain the presumption that our probiotic mixture made an effect on the serotonergic system without the mediation of the alterations of the SCFAs, thus, providing us the opportunity to hypothesize this to be the common way how the probiotic formulation can re-establish the behavioral alterations.

V.3. The effects of gastrointestinal microbiome modifications on fatty acids production

It is now undeniably known that the main short-chain fatty acids are produced by gut bacteria, furthermore, some fatty acids with longer, odd-numbered carbon atoms, such as C15:0 and C17:0, are also affected by the microbiome. Our current data also confirm these statements, and also revealed additional important saturated and unsaturated fatty acids, which are presumably linked to GI microbiome production.

Within the longer chain SATs, our finding revealed that the majority of these fatty acids are produced by intestinal bacteria, and not just those with odd-numbered carbon atoms as would be assumed based on former studies. The amount of all determined branched SATs was significantly reduced after ABx treatment, confirming the finding of a recent study that branched saturated fatty acids originate from bacterial degradation of proteins. The majority of the analyzed cMUFAs were decreased after ABx treatment suggesting that the GI microbiome is responsible for the production of these fatty acids. The investigated n-3 PUFAs and most of the n-6 PUFAs did not show a decrease in their concentration after ABx treatment, corroborating the common assumption that these fatty acids are greatly modulated by dietary intake. Recent studies suggested that concentrations of C20:2n-6 and C20:3n-6 were also influenced by gut bacteria. In this research we could demonstrate a similar but only partial relationship, that the microbiome is responsible for the production of C20:2n-6 and C20:3n-6 in the gut, and not the dietary intake.

Our aim was to restore the gut microbiota in a short time and to study the effect of this probiotic mixture on the detailed fatty acid spectrum. As we hypothesized, probiotic administration was able to initiate the reestablishment of bacterial populations and promoted changes in the concentration of several fatty acids after the ABx treatment. However, our mixture was unable to restore the concentration of each fatty acid to baseline. We hypothesized that in these cases (C6:0, C10:0, C11:0, C16:1n-9 and C16:1n-7) other bacterial genus species or additional microbial interactions may be involved. Nevertheless, most of the fatty acids, whose concentrations had been altered by the microbiome depletion, returned to their initial state after probiotic administration. Based on our results, we hypothesized that our probiotic mixture had a crucial role in the production of branched SATs, most SATs and even several MUFAs, and PUFAs, and therefore, by administering these strains after ABx treatment, a significant part of the fecal fatty acid spectrum could be restored.

In our study, we determined not only absolute but also relative concentration changes (weight percent ratio) of fatty acids, as this can also provide us useful information. Our results showed, that in the majority of fatty acids both concentrations decreased after two weeks of ABx treatment. The fact, that the weight percent ratios were also restored following the probiotic treatment means that the probiotic administration may have contributed to the changes in these proportions in the proper direction. Given that the weight percentage ratio demonstrates the relationship between fatty acids, the outcomes indicated that restoring the concentrations of most fatty acids were sufficient for normalizing this ratio.

The rats included in this study received the same food pellets, which contained the same fatty acid composition as determined by us. This consistency allowed us to monitor the changes in the fatty acids present in the rats' pellets during the treatments at each sampling time. Except the C18:2n-6, the fatty acids present in the rodents' chow showed decrease in their concentration after ABx treatment, and the subsequent probiotic administration restored them to their original state. The fact that the concentration of the vast majority of fatty acids continuously altered as a result of treatments affecting the gut microbiome provides preliminary evidence that the analyzed fatty acids, whose amounts changed due to the antibiotics treatment and were restored after the probiotic administration, were produced by the gut bacteria rather than derived from the diet.

VI. Summary

Taken together, the outcomes of experiments of one of the main objectives of this thesis work confirm that broad-spectrum antibiotics treatment during adulthood can induce antisocial behavior similar to that observed in the VPA autism animal model. To our best knowledge this study is among the first ones to demonstrate that specific probiotic mixture can restore the same type of antisocial behavioral phenomena in these two disparate animal models, developed by distinct mechanisms. Based on the present data, this probiotic formulation targets a common pathway with a non-SCFA dependent manner in both models, regardless of whether the antisocial behavior was formed by the significant reduction of the gut bacteria which generates changes along the microbiome-gut-brain axis, or by while the VPA animal model, which is a developmental model, that caused the same behavior through a completely different mechanism. Based on these findings, the results appear to confirm the hypothesis that the homeostatic balance of the GI microbiome has a huge impact on central nervous processes and also influences the social behavior.

The outcomes of the other main aims of this thesis is one of the first studies to analyze the role of gut bacteria on the detailed fatty acid composition of stool samples from adult rats. The results demonstrated that, in addition to SCFAs, a number of other saturated and unsaturated fatty acids as well as trans and branched-chain saturated fatty acids are determined by gut bacteria in rat stool. To the best of our knowledge, this research is among the first ones to demonstrate that ABx treatment reduces the concentration of fatty acids in rat feces and that a specific probiotic mixture can restore this disturbed fatty acid spectrum.
Thus, this study not only provides evidence for the role of intestinal bacteria in the production of specific fatty acids, but it also supports a new approach to treat disorders resulting from a shift in the ratio and concentration of fatty acids.

In conclusion, this research provides preliminary evidence that the gut microbiome appears to have a greater influence on both the quantitative and qualitative fatty acid composition of feces than previously understood. Additionally, certain specific probiotic bacterial combinations where shown to effectively restore the healthy fecal conditions, in addition, they also proved to be effective in alleviating disturbances in social behavior, indicating the potential therapeutic value of these probiotics.

This study provides preliminary evidence and possibly contribute to a better understanding of the complex and widespread regulatory system of the gastrointestinal microbiome. In the

future it might help develop the therapeutic potential of GI microbiome, more specifically some bacterial combinations, to cure or at least attenuate disorders resulting from alterations in the composition of fatty acids. Additionally, it could serve as a proper therapeutic agent to eliminate symptoms of antisocial behavior in individuals with ASD.

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VIII. List of publications

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Mintál K, Tóth A, Hormay E, Kovács A, László K, Bufa A, Marosvölgyi T, Kocsis B, Varga A, Vizvári Z, Cserjési R, Péczely L, Ollmann T, Lénárd L, Karádi Z. Novel probiotic treatment of autism spectrum disorder associated social behavioral symptoms in two rodent models. *Scientific reports* (2022) 12:5399 **IF:4.6; D1**

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