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A balanced omega-6/omega-3 polyunsaturated fatty acid diet suffices to prevent autism spectrum disorder symptoms in an environmental mouse model

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Abstract

Exploration of potential nutritional therapies in autism spectrum disorder (ASD), notably through omega-3 polyunsaturated fatty acid (n-3 PUFA) supplementation, have been explored but remain elusive as to their specific contribution to the phenotype and their potential in ameliorating cardinal symptoms of the disease. Here, we compared the effects of two diets that differ in their n-3 PUFA species on ASD symptoms in the valproic acid (VPA) mouse model. For this, pregnant C57BL/6J females were i.p. injected with VPA at embryonic day 12.5 (E12.5; 450mg/kg) and fed with either a balanced diet (n-3 bal) with alpha-linolenic acid (ALA) as the only n-3 PUFA source or a n-3 long-chain PUFA (LCPUFA) supplemented diet (n-3 supp) with docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) as the major n-3 PUFA species. Diets were provided starting E0, throughout lactation and on to the offspring after weaning through adulthood. Maternal and pup behaviors were investigated followed by social, motor and gait behavior in young adult offspring. Post-mortem investigations included cerebellar Purkinje cell (PC) count, liver and cerebellar fatty acid (FA) composition, inflammation markers' levels and microbiota composition. All experiments were performed separately on male and female offspring. Developmental milestones were delayed in the n-3 LCPUFA groups, whatever the treatment. VPA-exposed offspring did not show social deficits, stereotypies, or PC loss. Global activity and gait were altered by diet and treatment with sex differences. TNF-alpha cerebellar levels were slightly increased by n-3 LCPUFA supplementation, only in females. With both diets, VPA did not alter microbiota composition in male and female offspring nor cerebellar n-3 LCPUFA levels, except in females. Our results indicate that a balanced n-3/n-6 PUFA diet may suffice to protect from ASD symptoms and physiopathology, and that n-3 LCPUFA supplementation brings limited benefits in the VPA mouse model.

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by persistent deficits in communication and social interactions, and restricted repetitive behaviors, interests, or activities [1]. Imaging and post-mortem studies in ASD patients have identified the cerebellum as one of the major affected brain regions with reduction of its volume and decreased Purkinje cell (PC) number [2]. Similar findings were observed in both genetic and environmental animal models, where the cerebellar regions crus I and crus II involved in motor and cognitive functions were shown to be the most affected by PC loss, with sex differences [3–5]. Many comorbidities are also associated with ASD including inflammation, gastrointestinal and eating disorders [6,7]. There is currently no cure or preventive strategies for this disease other than symptomatologic.

Polyunsaturated fatty acids (PUFAs) from the omega-6 and omega-3 (n-6 and n-3) families are found in large quantities in brain cell membranes and are crucial for brain function and development [8,9]. Linoleic acid (LA, n-6) and alpha-linolenic acid (ALA, n-3) are essential fatty acids (FA) that cannot be synthetized by mammals and need to be provided through diet. Consumed LA and ALA are metabolized into long-chain (LC) PUFA, arachidonic acid (AA, n-6) and eicosapentaenoic acid (EPA, n-3)-docosahexaenoic acid

(DHA, n-3), respectively. Additional dietary supply from fatty fish is necessary to reach the recommended DHA and EPA brain levels [10].

PUFAs from the mother are crucial for the developing fetus brain during the perinatal period [9]. Studies showed that high n-6/n-3 ratio in this critical period may lead to aberrant brain lipid composition, metabolism, and signaling pathways [11,12]. This in turn may be associated with neurodevelopmental and psychiatric disorders [8,13,14]. Thus, n-3 PUFA supplementation during the perinatal period for prevention and at adulthood for correction of ASD have been studied in clinical and animal settings.

While several studies point to potential benefits of PUFA supplementation during the perinatal period, the exact contribution of n-6 and n-3 PUFAs species (i.e precursor *versus* long-chain) and the contribution of the n-6/n-3 ratio on neurodevelopmental disorders are not well defined. This, along with the conflicting results obtained in clinical ASD studies, prompt us to perform a large scale and a side-by-side comparison of two diets. The first is a balanced diet (n-3 bal) with linolenic acid (ALA) as the only n-3 PUFA source and the second is a n-3 long-chain PUFA (LCPUFA) supplemented diet (n-3 supp) with docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) as the major sources of n-3 PUFA species. This was performed in the valproic acid (VPA) mouse model of ASD, in males and females analyzed separately. Prior to this study, we had recently performed a full-scale analysis of this ASD mouse model on a wide range of behaviors with molecular and cellular correlates from early postnatal age to adulthood with special focus on motor behavior and cerebellar implications under a regular animal facility diet [3]. We showed major social deficits, stereotypies and cerebellar motor and gait disorders in the VPA ASD mouse model which were correlated with cerebellar regional PC loss in crus I and crus II. This allowed us to obtain a solid starting point to investigate n-3 LCPUFA supplementation effects on the ASD phenotype. Here, we extend our analysis to inflammation and FA parameters with microbiome, to determine their implication in dam and in male and female offspring behavioral, cellular and metabolic responses to differential diets.

Methods And Materials

Animals and treatment

Animal housing and experimental procedures were performed in accordance with the European Union directive (2010/63/EU) and validated by the ethics committee (Approval # 202002051628899). C57BL/6J mice (Charles River Laboratories) were housed in ventilated cages with access to food and water *ad libitum*. Room temperature was maintained at 23°C on a 12-h-light/-dark cycle (08:00–20:00).

A total of 337 mice were used in this study: 55 females and 44 males were used for mating, resulting in 120 female and 118 male offspring. From the first gestational day (E0) and throughout gestation and nursing, pregnant females were fed with a balanced isocaloric diet supplemented with n-3 LCPUFA (DHA and EPA) (n-3 supp) or not (n-3 bal) until weaning of their litter (SAFE, Augy, France). Both diets had a LA/ALA ratio of 6, meaning that they share the same amount of n-3 and n-6 precursors but not of n-3

LCPUFA (detailed description of diets are provided in Suppl. Table I). Pregnant females received a single i.p. injection of either VPA (450mg/kg, Sigma-Aldrich, P4543) or saline solution (NaCl 0.9%) at gestational day E12.5 as previously described [3,15]. After giving birth, dams were allocated to four experimental groups depending on prenatal treatment and diet: SAL without n-3 LCPUFA (SAL/n-3 bal), VPA n-3 without LCPUFA (VPA/n-3 bal), SAL n-3 LCPUFA-supplemented (SAL/n-3 supp) and VPA n-3 LCPUFA-supplemented (VPA/n-3 supp) (timeline and experimental procedures are detailed in Suppl. Figure 1). Male and female offspring were separated at weaning (postnatal day 28, P28) into one of the following 8 groups for behavior analysis: 29 males and 39 females SAL/n-3 bal, 36 males and 27 females SAL/n-3 supp, 27 males and 28 females VPA/n-3 bal, and 26 males and 26 females VPA/n-3 supp. For microbiota, FA profile and inflammation markers, the same animals were used: 5 randomly selected animals per group, with a maximum of 2 animals from the same litter and sex.

Maternal and offspring behavior procedures

Maternal behavior experiments were performed on SAL/n-3 bal (n = 15), SAL/n-3 supp (n = 14), VPA/n-3 bal (n = 13), and VPA/n-3 supp (n = 12) groups. Dam performances on pup retrieval were assessed at P9. For this test, both the litter and the dam were removed and separated from the home cage for 5 minutes before being placed back. Dam behavior directed towards the pups, or the environment was recorded for 15 minutes and analyzed on Solomon Coder (András Péter, Keele, UK).

Developmental milestones on all offspring were assessed by measuring righting reflex at P9, P11 and P13 as well as eye opening from P12 to P16. Spontaneous activity in the cylinder was recorded at P48 for grooming and rearing scoring using Solomon Coder (András Péter, Keele, UK) as previously described [3,16]. Spatial, temporal, and kinematic gait parameters were analyzed during spontaneous walk at P49 using an automated gait analysis system (Gaitlab, Viewpoint, France) as previously described [3]. Social interaction was assessed between P50 and P60 using the three-chamber test (3-CT) as previously described [3,17].

Tissue processing, immunohistochemistry and stereology counting

Animal perfusion, brain retrieval, tissue sectioning and cresyl violet/neutral red staining were performed as previously described [4]. Stereological estimates of Purkinje cell (PC) number within crus I and II cerebellar regions were obtained using the optical fractionator method (Mercator Software, Explora Nova, France) and systematic random sampling as previously described [4] in the following groups: [Crus I: SAL/n-3 bal (males n = 11 and females n = 13), SAL/n-3 supp (males n = 11 and females n = 7), VPA/n-3 bal (males n = 10 and females n = 11) and VPA/n-3 supp (males n = 10 and females n = 9)] and [Crus II: SAL/n-3 bal (males n = 11 and females n = 12), SAL/n-3 supp (males n = 12 and females n = 9), VPA/n-3 bal (males n = 10 and females n = 11) and VPA/n-3 supp (males n = 10 and females n = 9].

Fecal microbiome analysis

Fecal samples were collected from both the dam through lactation period (P15, P20 and P22) and the litter after weaning (P36, P41 and P43). Samples were conserved at -80°C in nucleic acid conservative buffer until DNA extraction (RNA Protect, Qiagen, Venlo, The Netherlands). Wet-lab and bioinformatic analysis were performed as previously described [18,19]. After verification on rarefaction curve, rarefaction (subsampling to 5000 sequences per sample) was performed before determination of alpha diversity metrics (Chao1's and Shannon's indexes), and beta diversity metrics (Bray-Curtis dissimilarity and weighted UniFrac [20]). ANCOM was used to identify differentially abundant genera among the groups [21]. Raw data are available under the SRA accession numbers (SRR19906240 to SRR19906299).

Analysis of inflammation makers by qPCR

RNA expression of major genes implicated in inflammatory process was investigated as previously described [22]. Briefly, RNA from hemi-cerebellum of dams and male and female offspring was extracted using TRIzol extraction kit (Invitrogen, Life Technologies, Saint-Quentin-Fallavier, France). Purity and concentration of RNA were determined using a Nanodrop 1000 spectrophotometer (Nanodrop technologies, Wilmington, DE, USA) and a bioanalyzer (Agilent, Les Ulis, France). Reverse transcription was performed on one or two micrograms of RNA by Superscript IV (Invitrogen, Life Technologies, Saint Aubin, France). TaqMan® specific primers were used to amplify genes of interest as previously described [22] with a focus on tumor necrosis factor alpha (TNF-alpha, Mm00443258_m1), transforming growth factor-beta 1 (TGF-beta 1, Mm01178820_m1) and Arginase 1 (Arg1, Mm00475988_m1). The housekeeping gene was beta-2-microglobulin (B2M, Mm00437762_m1). Fluorescence was determined on a LightCycler® 480 instrument II (Roche, La Rochelle, France). Data were analyzed using the comparative threshold cycle (Ct) method and results were expressed as relative fold change to control target mRNA expression.

Analysis of fatty acids in cerebellum and liver

FAs from the liver and the cerebellum were analyzed as previously described [23–25]. Briefly, liver and cerebellum FAs were extracted according to the Folch's method [26], FAs were transmethylated according to Morrison and Smith's method [27] and FA methyl esters (FAMEs) were analyzed on a FOCUS GC gas chromatograph (Thermo Electron Corporation) equipped with a split injector and a flame ionization detector. FAMEs were identified by making a comparison with commercial standards. FA composition is expressed as the percentage of total FAs.

Statistical analyses

For experiments related to behavior, data are expressed as mean ± Standard Error of the Mean while cellular and metabolic experiments, data are expressed as median ± min to max and analyzed using GraphPad Prism-9 software (La Jolla, California, USA). Data were analyzed using two-way or three-way analysis of variance (ANOVAs) followed by Tukey post-hoc multiple comparisons test. For all analyses, a p value < 0.05 was considered significant. Raw data and detailed statistical analysis on all groups are available upon request.

Results

Maternal behavior is not affected by either VPA treatment or diet

VPA-exposed juvenile mice produce aberrant patterns of isolation stress-induced ultrasonic vocalizations [28]. This may influence in return maternal behavior, which is key to normal social and motor development of offspring [29]. Here, we assessed various parameters related to maternal behavior following pup separation in relation with treatment and diet (Suppl. Figure 2). Two-way ANOVA analysis indicated no differences between experimental groups, whatever the treatment or diet in maternal care, nesting and stress related behaviors. In summary, our results indicate that VPA treatment did not affect maternal behavior when mothers were fed either n-3 LCPUFA or its precursors only.

Developmental milestones of offspring are delayed by n-3 LCPUFA supplementation

We have recently shown that prenatally VPA-exposed mice exhibit significant early postnatal behavioral impairments [3]. Here, we investigated eye opening time-period and righting reflex from P9 to P16 in relation to treatment and diet (Fig. 1). Eye opening was delayed in both male and female SAL/n-3 supp mice compared to SAL/n-3 bal mice at P13 and only in males at P14. Eye opening was also delayed in VPA/n-3 supp females compared to VPA/n-3 bal females at P13 and P14. In addition, SAL/n-3 supp males showed an aberrant righting reflex at P9 and P13 compared to the SAL/n-3 bal group and compared to the VPA/n-3 supp group at P13 only. Similar differences were found in females at P11 but not at P13.

These results indicate that n-3 supp diet affected sensorimotor development in conjunction with VPA treatment and sex of the animals.

No major behavioral or cellular alterations following VPA treatment whatever the diet

Among others, we have reported that prenatally VPA-exposed mice consistently show reduced social behavior in the 3-CT test with mice under a regular animal facility diet [3,15,30]. Here, using the same experimental procedures in the same housing conditions, both diets prevented the VPA-induced sociability impairments in male and female young adult mice (Fig. 2A). Three-way ANOVA showed that all groups have normal social abilities. A treatment effect was found in VPA/n-3 supp males that were slightly more social than the SAL/n-3 supp group.

Stereotyped behavior has often been reported in various genetic and environmental animal models of ASD [2–5]. This behavior is reminiscent of repetitive behaviors in ASD patients and constitutes one of the major symptoms used in the diagnosis of ASD [1]. We have previously shown that prenatal exposure to

VPA significantly increases stereotyped behavior in both males and females as compared to controls [3]. Here, we found no differences in grooming parameters between groups, except on grooming duration in females where VPA treatment had a significant effect but no subsequent differences between groups were found (Fig. 2B). However, both VPA/n-3 bal male and female mice showed an increased frequency and duration of rearing behavior compared to both SAL/n-3 bal and VPA/n-3 supp mice (Fig. 2C). No differences in rearing between VPA/n-3 supp *versus* SAL/n-3 supp groups or in SAL/n-3 bal *versus* SAL/n-3 supp groups were found.

Although gait impairments are not yet considered in the diagnosis criteria of ASD, they have often been reported in both ASD patients and animal models [31,32]. We have previously shown significant gait deficits in genetic and environmental ASD animal models, including the VPA model [2–5]. Here we show moderate gait deficits, which were for the most part observed in the VPA/n-3 supp male group as compared to the SAL/n-3 supp group (Fig. 3). For instance, dynamic and temporal parameters such as stride length and swing time were affected. Morphological parameters such as paw width, paw length and paw area were also altered by both treatment and diet. In females, only a few parameters were affected, such as kinematic-related fore and hindlimbs base of support. VPA/n-3 bal females showed a decreased base of support compared to the VPA/n-3 supp group on both forelimbs and hindlimbs, and additionally to SAL/n-3 bal females for forelimbs only.

Taken together, these results indicate that both diets were able to reduce if not compensate for ASD related social, motor and gait symptoms as only moderate to no differences between groups were observed, whatever the treatment, diet, or sex.

We have previously shown significant PC number decreases in the crus I or crus II cerebellar areas in both environmental and genetic ASD animal models [3–5]. Here, we examined PC number in crus I and crus II in all experimental groups (Suppl. Figure 3). Two-way ANOVA analysis showed no treatment or diet effect in either males or females.

These results indicate that both n-3 bal and n-3 supp diets protected from PC loss induced by VPA in males and females alike.

Diet, but not VPA treatment, differentially alter n-3 and n-6 LCPUFAs levels in the liver, but not in the cerebellum

We assessed the FA profile of the liver and the cerebellum, a cerebral region high in terms of DHA content and which is sensitive to changes in FA composition [33]. Analyses were performed longitudinally to compare dam and offspring FA profiles. Given that n-3 LCPUFA supplementation was provided through diet, we analyzed, among others, levels of n-3 and n-6 LCPUFA, DHA and AA (Fig. 4 and Suppl. Figure 4). In the liver, in all n-3 LCPUFA supplemented groups, whether dams, males or females, a significant increase (up to 3-fold) in DHA levels was found compared to n-3 balanced groups. In the cerebellum, DHA

levels also increased in the VPA/n-3 supp compared to VPA/n-3 bal, but only moderately (+ 25% approximately) and only in female offspring. Interestingly, no group differences in DHA cerebellar levels were found in dams and male offspring. The AA levels decreased in SAL/n-3 supp and VPA/n-3 supp groups compared to SAL/n-3 bal and VPA/n-3 bal groups respectively both in the liver and the cerebellum and in males and females. However, dam AA levels in the liver increased in the VPA/n-3 bal compared to both SAL/n-3 bal and VPA/n-3 supp.

These results indicate a major effect of n-3 LCPUFA supplementation on DHA and AA levels within the liver but not within the cerebellum where DHA levels were equivalent, whatever the treatment and diet.

Moderate to no alterations in inflammatory markers in conjunction with diet and treatment

N-3 PUFAs are associated with anti-inflammatory processes and could influence behavior output [9]. Here, we investigated several inflammation markers in the offspring cerebellum: TNF-alpha, TGF-beta, and ARG. No differences were found between groups for TGF-beta and ARG, whatever the sex. However, TNF-alpha expression increased slightly in SAL/n-3 supp females compared to SAL/n-3 bal females. While VPA treatment increased TNF-alpha levels in males, no subsequent differences between groups were found (Fig. 5).

In brief, moderate to no effects of diet or treatment on inflammation markers were observed.

Microbiota inter- and intra-diversity and bacteria relative abundance are not affected by diet

Microbiota has been shown to be influenced by *in utero* VPA treatment on offspring and by n-3 PUFA supplementation or deficiency [34–36]. Using a n-3 balanced or n-3 LCPUFA supplemented diet, we found no differences in alpha-diversity in either dams or offspring. Interestingly, offspring beta-diversity was not different across groups, whereas dam beta-diversity increased by VPA treatment, whatever the diet, with VPA n-3 suppl dams being the most dissimilar (Fig. 6A and Suppl. Figure 5–7). Additionally, in dams, but not in offspring, relative abundance of *Bacteroidetes* and *Actinobacteria* was increased in VPA/n-3 supp group compared to SAL/n-3 supp and VPA/n-3 bal group (Fig. 6B and Suppl. Figure 8). No differences were found in offspring's microbial composition, except in males, where Bray-Curtis dissimilarity analysis showed that the VPA/n-3 supp group has a higher microbial composition compared to SAL/n-3 supp group (Suppl. Figure 6). These results show no major differences between diets on the offspring microbiota, whatever the treatment.

Discussion

In this study, we investigated the potential benefits of adding n-3 LCPUFA to a balanced diet starting from the perinatal period to adulthood on a multiplicity of social, motor and gait behaviors as well as on cerebellar cellular, molecular, and metabolic correlates in both male and female VPA mouse model of

ASD. We found that adult VPA-exposed animals, whatever the diet, were not showing social deficits, stereotypies or cerebellar PC loss, all major hallmark of ASD. Developmental milestones, gait and inflammatory profiles were only slightly affected by diet in conjunction with VPA prenatal exposure. Microbiota composition was not altered in relation with diet and treatment. Additionally, cerebellar DHA levels were somewhat equivalent, whatever the diet, while liver DHA levels dramatically increased with a n-3 LCPUFA supplementation. Using dams and their offspring, we were also able to perform longitudinal studies on several parameters. We found that while maternal behavior was not altered by either treatment or diet, dams exhibited a change in their microbiota composition and increased n-6 LCPUFA liver levels in conjunction with VPA treatment.

The n-3 LCPUFA supplemented groups exhibited a slight delay in both righting reflex and eye opening, in accordance with previous studies reporting adverse consequences in postnatal development after maternal dietary n-3 LCPUFA supplementation or deficiency during gestation and lactation [37–40]. Contrary to the common misconception that elevated levels of n-3 LCPUFAs are necessary beneficial, a change in the n-6/n-3 PUFA ratio, irrespective of its direction, was reported to be deleterious during development, without major consequences at adulthood, although this also depends on the animal model [41,42]. Indeed, in both the BTBR and C57BL/6J mouse strains, body weight was decreased following perinatal diet intervention whereas in the Fmr1 KO mouse, body weight was increased [41,42]. Here, ALA from the n-3 balanced diet did not lead to developmental delays and was even able to normalize both righting reflex and eye-opening scores in VPA-exposed animals. Metabolic studies on this matter may help unravel the mechanisms behind this developmental delay.

Deficits in social preference or social novelty and increased grooming behavior are repeatedly found in several animal models of ASD including the VPA model under a standard diet, as we also reported recently [3–5]. Here, we found that both diets, n-3 balanced with and without n-3 LCPUFA, protected against sociability deficits and stereotypy anomalies, two major ASD-associated symptoms. To our knowledge, this study is the first to investigate n-3 PUFA precursor effects on social behavior since the vast majority of studies compared n-3 LCPUFA supplementation and deficiency, where n-3 LCPUFA supplementation was shown to alleviate stereotypies and social behavior impairments [43].

We have previously shown, in the same experimental settings, that rearing, which represents global activity, decreased in VPA-exposed animals fed with a standard diet [3]. Here, the n-3 balanced diet increased rearing behavior in VPA-exposed animals compared to controls and this was normalized with n-3 LCPUFA supplemented diet. In physiological conditions, high n-3 LCPUFA supplementation for 3 weeks after weaning reduces rearing [44], whereas in depression and anxiety models, which are ASD comorbidities, opposite results are found [45].

Our previous work on several environmental and genetic ASD animal models fed with a regular diet consistently showed motor and gait impairments [3–5]. Gait is seldom explored in these models even though ASD patients exhibit an irregular walk and balance difficulties associated with cerebellar

dysfunction [46]. Here, we showed that diet had a differential effect on ASD VPA male and female mice. The n-3 LCPUFA supplementation ameliorated gait parameters in females, with an increased hindlimb base of support, suggesting better stability, whereas VPA-exposed males with n-3 LCPUFA supplementation displayed dynamic, temporal and morphological impairments compared to controls, albeit of a lower magnitude than what we observed with animals under a regular diet [3]. These results suggest moderate sex-dependent gait deficiencies in conjunction with n-3 LCPUFA supplementation, possibly resulting from metabolic differences between males and females hypothesized to be estrogen-related [47].

Crus I and crus II cerebellar regions are involved in both cognitive and motor functions, which make them a target of choice in ASD physiopathology [2,48]. A decrease in PC number has been widely reported in both ASD patients and in animal models, including the VPA mouse model under a regular animal facility diet [3,5,49–52]. Here, we showed that PC cell number in these regions was not altered between groups, whatever the treatment, diet, or sex. These findings fit with our main hypothesis, which is that the n-3 balanced diet is sufficient to protect from ASD behavioral symptoms and cellular correlates and that supplementation with n-3 LCPUFA does not yield additional benefits in these conditions.

Furthermore, to determine whether n-3 PUFA supplementation is associated with higher n-3 PUFA levels in the periphery or in the central nervous system, we investigated FA profiles in the liver and the cerebellum. The n-3 LCPUFA supplemented diet was highly supplemented in DHA, whereas the n-3 balanced diet contained only n-3 and n-6 precursors (ALA and LA), with shared LA/ALA ratio of 6 in the two diets. Thus, our lipid analysis focused on n-3 and n-6 LCPUFA, DHA and AA respectively. As expected, n-3 LCPUFA supplementation resulted in a drastic decrease in liver and cerebellum AA levels in both males and females. However, we found increased DHA levels in the liver in all groups. In the cerebellum, there was a diet effect with increased DHA levels in the VPA female group, but not in males where equivalent DHA levels were found. This indicates that beyond a n-3 balanced diet, a n-3 LCPUFA supplementation does not further increase cerebellar DHA levels, at least in the male groups. This suggests that this diet would bring limited if any benefit to the brain metabolism, translating into limited to no additional beneficial effects on ASD behavioral symptoms. These findings are in line with another study where a n-3 balanced diet with ALA as the only source of n-3 PUFA sufficed to increase DHA and decrease AA levels in the cortex and protected from deficits in emotional behavior in adult and old CD1 mice (2–5 months and 19–23 months) as compared to a n-3 deficient diet [53]. Previous studies have investigated the role of n-3 and n-6 LCPUFA on inflammation and concluded that high DHA brain levels are linked to an anti-inflammatory profile whereas high AA brain levels are correlated with a pro-inflammatory profile [54–56]. We found that TNF-alpha levels increased slightly in SAL/n-3 supp females only. Taken together, these results on FA profiles and inflammation highlighted sex differences and female sensitization to n-3 LCPUFA supplementation.

ASD patients suffer from gastrointestinal issues (GI) hypothesized to result from a microbiota dysbiosis, i.e. a microbial composition imbalance [57,58]. Differences in alpha and beta-diversity and an imbalance in

Bacteroidetes and *Firmicutes* have been consistently reported in the VPA ASD mouse model [34,59]. The n-3 LCPUFA supplementation was shown to contribute to microbiota diversity and homeostasis [36,60], but studies on ALA effects are lacking. Here, we found no differences in alpha-diversity, beta-diversity, or phyla abundance in either male or female offspring, whatever the sex, treatment, or diet. This further consolidates our hypothesis that n-3 LCPUFA supplementation with DHA and EPA brings only limited benefits compared to a balanced n-3/n-6 diet, and that ALA suffices to protect from VPA-induced ASD symptoms and from cellular, biochemical, and metabolic consequences in our conditions.

One of the strengths of this study is the global and longitudinal approach where, in addition to male and female offspring analyzed separately, we also investigated treatment and diet influences on dams' maternal behavior, liver and cerebellum FA profiles and microbiota composition. Maternal care received by the pups during the first postnatal weeks can affect their behavior in adulthood [61,62] and in our hands, we did not find any differences on maternal behavior between the dams' groups, whatever the treatment or diet. These findings align with those of another study, where VPA treatment did not affect maternal behavior [63]. However, we found more drastic differences due to treatment and diet interaction in dams than in offspring pertaining to maternal FA profiles and microbiota composition. In fact, VPA-exposed dams with the n-3 balanced diet exhibited increased AA liver levels, which were normalized with the n-3 LCUPFA supplementation diet. In addition, *Bacteroidetes* and *Actinobacteria* proportion in VPA/n-3 supp dams increased, as did the beta-diversity in this experimental group. Dams may be more sensitive to diet changes as they were fed with a regular diet before gestation, whereas offspring were given the same diet from embryonic stage to adulthood.

Taken together, our findings indicate that n-3 LCPUFA supplementation brings only limited benefits to the ASD phenotype in the VPA mouse model when compared with a n-3 balanced diet. These beneficial effects were evidenced at the behavioral, cellular, and molecular levels, in both sexes, although females seem to be somewhat more sensitive to n-3 LCPUFA supplementation. Additional investigations are warranted, aiming at deciphering the underlying mechanisms of dietary effects on ASD brains. They also need to consider sex and age differences, two parameters seldom investigated at least in preclinical settings, where experiments are performed mostly in young adult males under a regular diet.

Declarations

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Figures

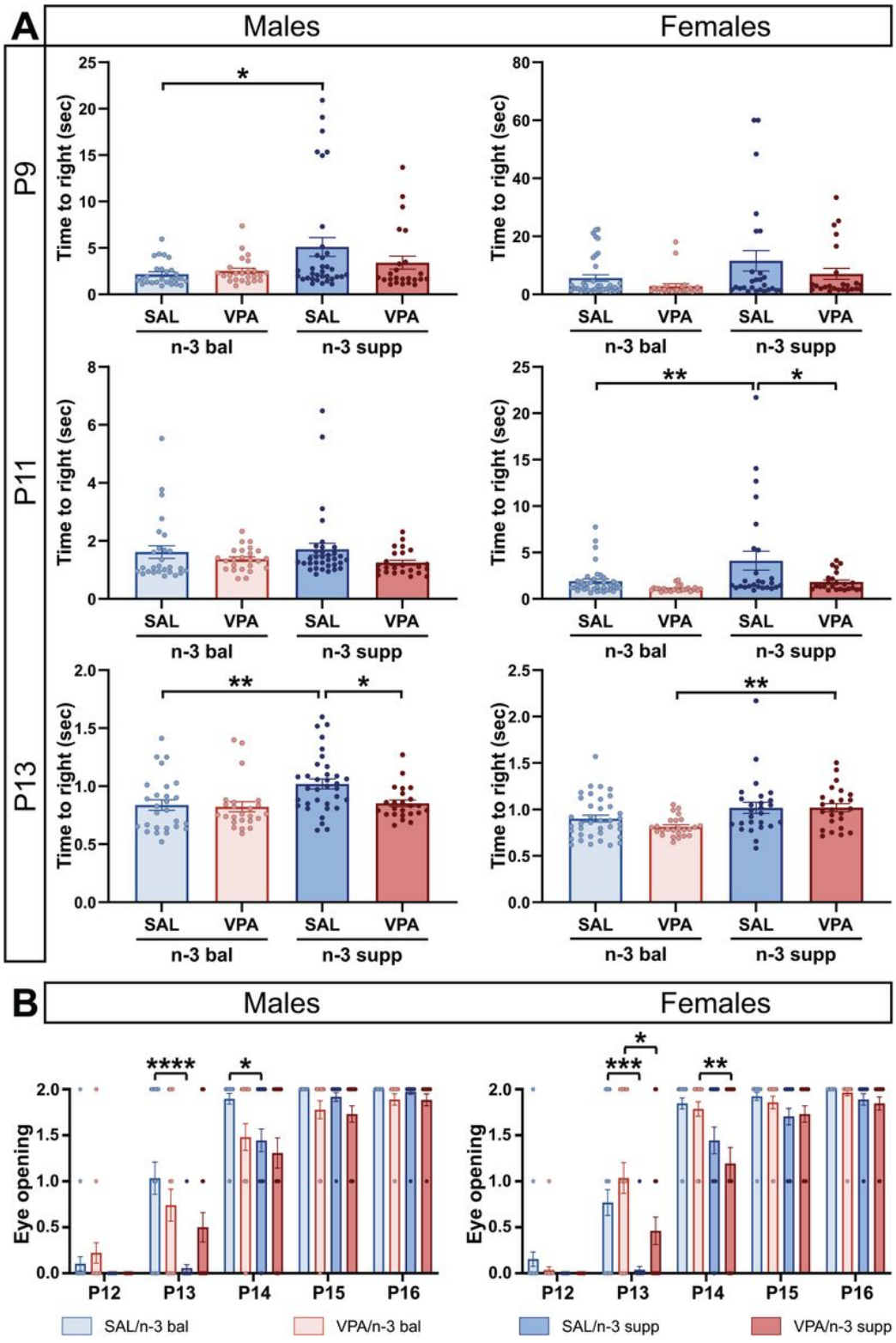

Figure 1

Developmental milestones of offspring are delayed by n-3 LCPUFA supplementation. (A) Righting reflex time at P9 (top), P11 (middle) and P13 (bottom) in both males (left) and females (right). n = 27 (SAL/n-3 bal male), 24 (VPA/n-3 bal male), 34 (SAL/n-3 supp male), 24 (VPA/n-3 supp male), 38 (SAL/n-3 bal female), 25 (VPA/n-3 bal female), 26 (SAL/n-3 supp female) and 24 (VPA/n-3 supp female) mice. **(B)** Eye opening score from P12 to P16 in both males (left) and females (right). n = 29 (SAL/n-3 bal male), 27

(VPA/n-3 bal male), 36 (SAL/n-3 supp male), 26 (VPA/n-3 supp male), 39 (SAL/n-3 bal female), 28 (VPA/n-3 bal female), 27 (SAL/n-3 supp female) and 26 (VPA/n-3 supp female) mice. Data are expressed as mean ± SEM and were analyzed through a two-way ANOVA followed by Tukey post-hoc multiple analysis. *p< .05, **p < .01, ***p < .001.

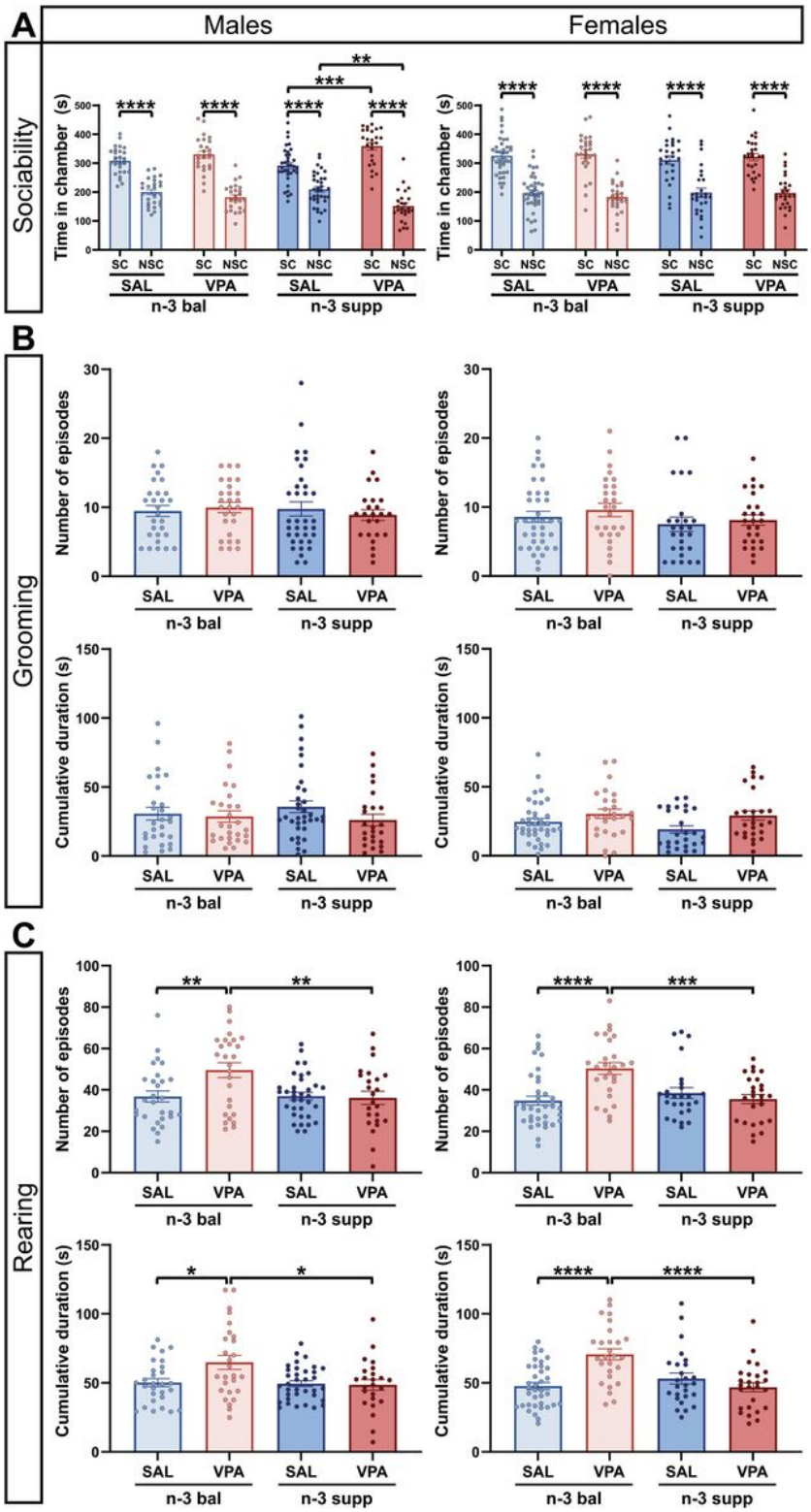

Figure 2

Offspring social, and grooming behavior are not altered by VPA following n-3 balanced and n-3 LCPUFA supplemented diets while rearing behavior is affected by a n-3 LCPUFA supplementation. (A) Phase II from the 3-CT in both males (left) and females (right females). n = 27 (SAL/n-3 bal male), 25 (VPA/n-3 bal male), 35 (SAL/n-3 supp male), 25 (VPA/n-3 supp male), 39 (SAL/n-3 bal female), 27 (VPA/n-3 bal female), 27 (SAL/n-3 supp female) and 26 (VPA/n-3 supp female) mice. Data are expressed as mean ± SEM and were analyzed through a three-way ANOVA followed by Tukey post-hoc multiple analysis. **p*< .05, ***p*< .01, ****p* < .001. SC: social chamber, NSC: non-social chamber. **(B)** Number of grooming episodes and cumulative grooming duration in both males (left) and females (right). **(C)** Number of rearing episodes and cumulative rearing duration in both males (left) and females (right). n = 28 (SAL/n-3 bal male), 27 (VPA/n-3 bal male), 35 (SAL/n-3 supp male), 24 (VPA/n-3 supp male), 37 (SAL/n-3 bal female), 27 (VPA/n-3 bal female), 26 (SAL/n-3 supp female) and 26 (VPA/n-3 supp female) mice. Data are expressed as mean ± SEM and were analyzed through a three-way ANOVA for 3-CT or two-way ANOVA for grooming and rearing behavior, followed by Tukey post-hoc multiple analysis. **p*< .05, ***p* < .01, ****p*< .001.

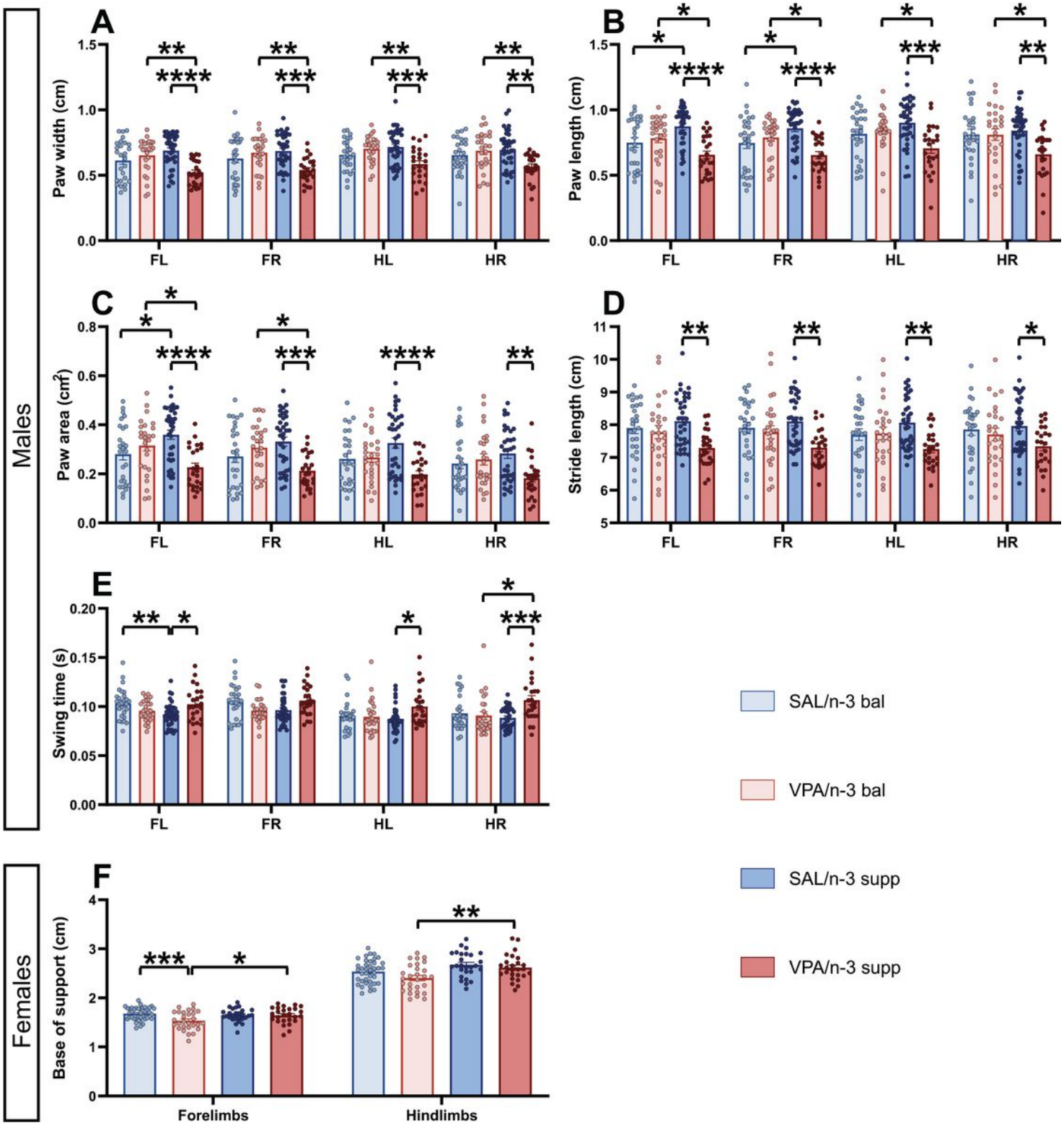

Figure 3

Offspring gait analysis exhibited sex differences in relation with diet. **(A)** Paw width in males. **(B)**Paw length in males. **(C)** Paw area in males. **(D)** Stride length in males. **(E)** Swing time in males. n = 27 (SAL/n-3 bal male), 26 (VPA/n-3 bal male), 36 (SAL/n-3 supp male), 24 (VPA/n-3 supp male) mice. **(F)** Fore and Hindlimbs Base of Support in females. n=39 (SAL/n-3 bal female), 28 (VPA/n-3 bal female), 27 (SAL/n-3 supp female), 26 (VPA/n-3 supp female) mice. Data are expressed as mean ± SEM and were

analyzed through a two-way ANOVA followed by Tukey post-hoc multiple analysis. *$p < .05$, **$p < .01$, ***$p < .001$.

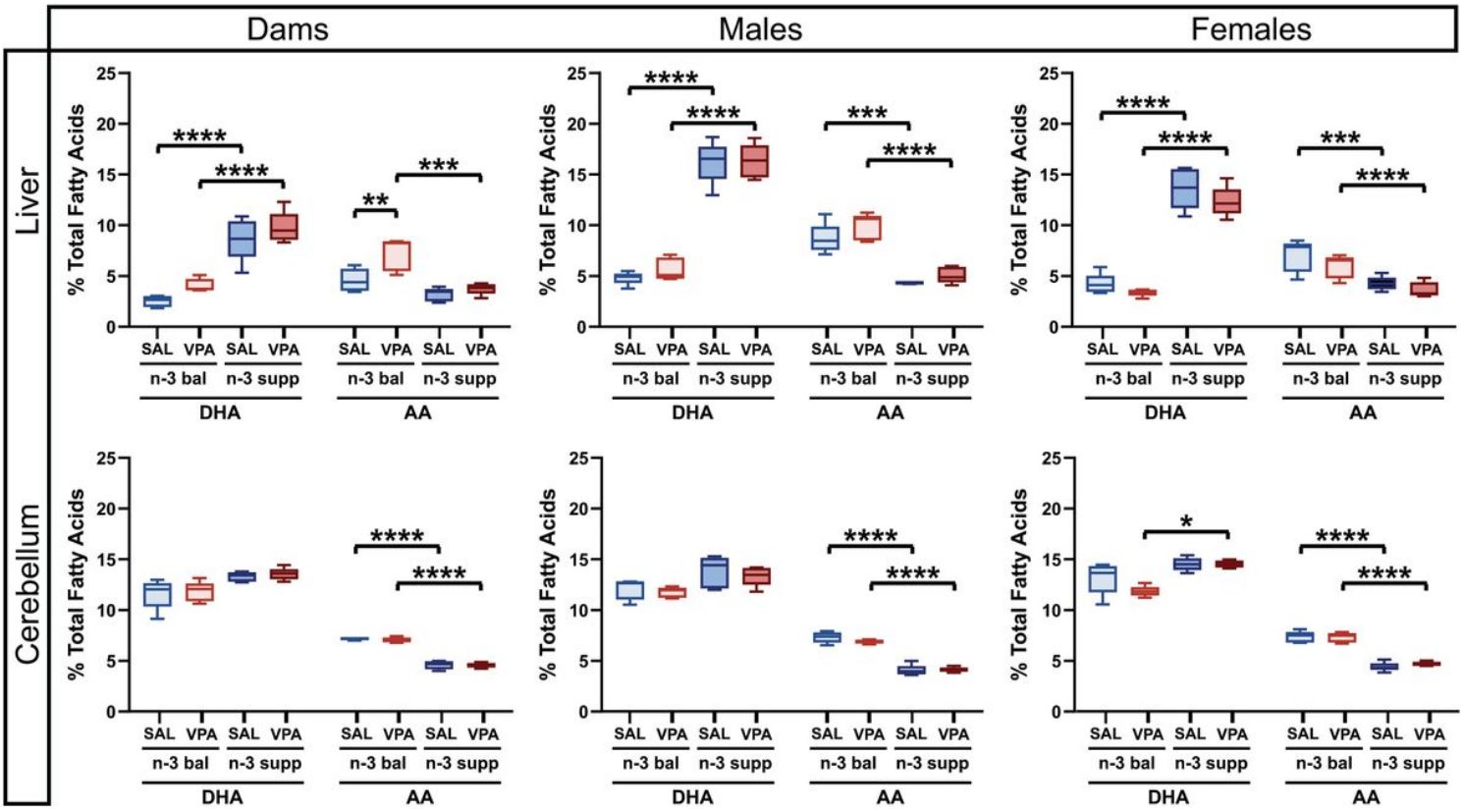

Figure 4

n-3 and n-6 LCPUFA profiles are modified in the liver, but not in the cerebellum, in relation with diet. Liver DHA and AA levels (top) in dams (left), offspring males (center) and females (right). n = 5 mice per group except 4 on SAL/n-3 supp male AA levels. Cerebellum DHA and AA levels (bottom) in dams (left), offspring males (center) and females (right). n = 5 mice per group except 4 on SAL/n-3 bal male and VPA/n-3 supp female AA levels. Data are expressed as median and min to max and were analyzed through a two-way ANOVA followed by Tukey post-hoc multiple analysis. *$p < .05$, **$p < .01$, ***$p < .001$.

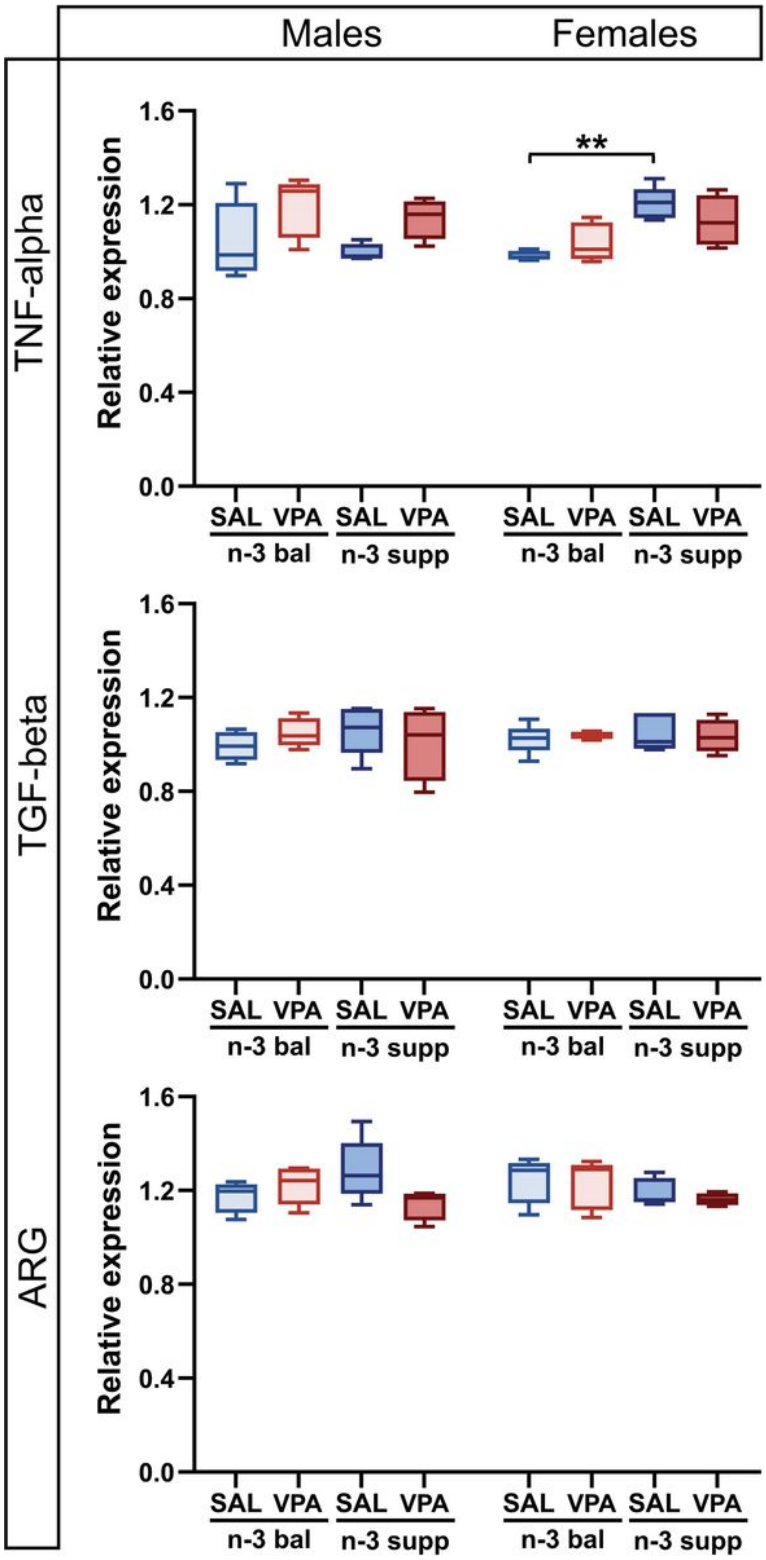

Figure 5

Moderate to no alteration in inflammatory markers in relation with diet and treatment. TNF-alpha cerebellar levels (top) in both males and females. n = 5 (SAL/n-3 bal male), 5 (VPA/n-3 bal male), 4 (SAL/n-3 supp male), 4 (VPA/n-3 supp male), 4 (SAL/n-3 bal female), 5 (VPA/n-3 bal female), 5 (SAL/n-3 supp female) and 5 (VPA/n-3 supp female) mice. TGF-beta cerebellar levels (middle) in both males and females. n = 5 (SAL/n-3 bal male), 5 (VPA/n-3 bal male), 5 (SAL/n-3 supp male), 4 (VPA/n-3 supp male), 5

(SAL/n-3 bal female), 5 (VPA/n-3 bal female), 5 (SAL/n-3 supp female), 4 (VPA/n-3 supp female) mice. ARG cerebellar levels (bottom) in both males and females. n = 5 (SAL/n-3 bal male), 5 (VPA/n-3 bal male), 5 (SAL/n-3 supp male), 4 (VPA/n-3 supp male), 5 (SAL/n-3 bal female), 5 (VPA/n-3 bal female), 5 (SAL/n-3 supp female), 4 (VPA/n-3 supp female) mice. Data are expressed as median and min to max and were analyzed through a two-way ANOVA followed by Tukey post-hoc multiple analysis. *$p < .05$, **$p < .01$, ***$p < .001$.

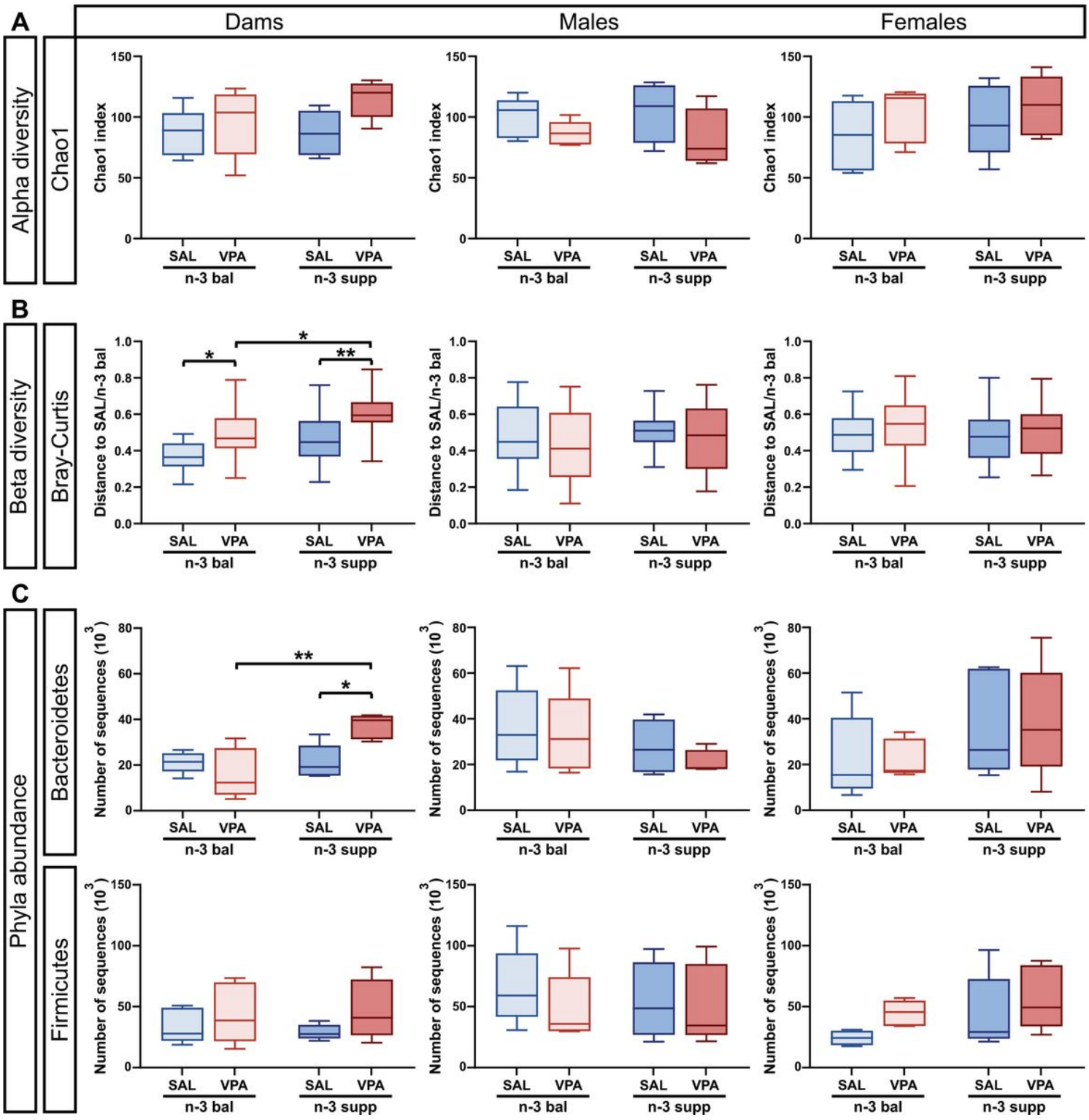

Figure 6

VPA does not alter offspring microbiota inter- and intra-diversity nor offspring microbiota relative abundance under both n-3 balanced and n-3 LCPUFA supplemented diets. Microbial diversity: **(A)** Chao1 index (first line) in dams (left), offspring males (center) and females (right). n = 5 mice per group. **(B)** Bray-Curtis index (third line) in dams (leftl), offspring males (center) and females (right). n = 10 (SAL/n-3 bal dam), 25 (VPA/n-3 bal dam), 25 (SAL/n-3 supp dam), 25 (VPA/n-3 supp dam), 10 (SAL/n-3 bal male), 25 (VPA/n-3 bal male), 20 (SAL/n-3 supp male), 25 (VPA/n-3 supp male), 10 (SAL/n-3 bal female), 25 (VPA/n-3 bal female), 25 (SAL/n-3 supp female) and 25 (VPA/n-3 supp female) mice. **(C)** Phyla abundance: Bacteroidetes abundance (first line) in dams (left), offspring males (center) and females (right). n = 5 mice per group except 4 in SAL/n-3 supp and VPA/n-3 supp male. Firmicutes abundance (second line) in dams (left), offspring males (center) and females (right). n = 5 mice per group except 4 in SAL/n-3 supp male and SAL/n-3 bal female. Data are expressed as median and min to maxand were analyzed through a two-way ANOVA followed by Tukey post-hoc multiple analysis. *$p < .05$, **$p < .01$, ***$p < .001$.

Supplementary Files

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