APOE genotype and postnatal chlorpyrifos exposure modulate gut microbiota and cerebral short-chain fatty acids in preweaning mice.

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Abstract

The gut microbiota comprises a large number of microorganisms, whose composition can be modified by genetic and environmental factors. The host's genetic background, including the different isoforms of the apolipoprotein E (*APOE*) gene, can exert an influence over microbiota composition. Exposure to the widely-used pesticide chlorpyrifos (CPF), can lead to dysbiosis and alter the levels of metabolites produced by the microbiota, such as short-chain fatty acids (SCFAs). This study was aimed at assessing the contribution of the *APOE* genotype and early exposure to CPF on gut microbiota and SCFA in brain. For it, C57BL/6, apoE3- and apoE4-TR mice were postnatally exposed to CPF. Microbiota in the gut and SCFA in the brain were assessed at PND 15 after CPF exposure. Differences between genotypes at different taxonomic levels were found, A. muciniphila presented greater abundance in APOE4 genotype, but was reduced by CPF exposure. APOE and CPF influenced cerebral SCFAs, with APOE3 genotype showing the highest levels of acetic, propionic and butyric acids and CPF exposure inducing the highest levels of isovaleric and 4-methylvaleric acids. These results provide further knowledge about gut microbiota and cerebral SCFAs composition at early ages and their modulation by APOE and postnatal CPF exposure.

Keywords:

Chlorpyrifos, APOE, Gut microbiota, short-chain fatty acids, *Akkermansia muciniphila*

1. - Introduction

In recent years there has been increasing interest in the functions of the gut microbiota, i.e. the large number of microorganisms residing in the intestine in a symbiotic relationship with their host (Gomes et al., 2018; Young and Schmidt, 2009). These microorganisms are involved in different functions such as digestion, metabolism (Joyce and Gahan, 2014) and immune function (Kamada et al., 2013), and they can influence the health status in general (Clemente et al., 2012; Sekirov et al., 2010). The gut microbiota undergoes a number of changes from birth (Bäckhed et al., 2015) and achieves a relatively stable composition by adulthood (Palmer et al., 2007). Nevertheless, it can be disrupted by several factors that may lead to dysregulation or dysbiosis (Hawrelak and Myers, 2004), with early postnatal ages being the most sensitive period to external factors. Microbiota dysbiosis has been linked to several diseases, such as autism (Needham et al., 2018), obesity and diabetes (Lee et al., 2019), cardiovascular affections (Katsimichas et al., 2019), allergy and asthma (Fujimura and Lynch, 2015), as well as neurodegenerative diseases (Marizzoni et al., 2017).

Microbiota composition can be affected by changes in diet (Makki et al., 2018; Sonnenburg and Bäckhed, 2016; Turnbaugh et al., 2008), drugs (Lange et al., 2016) and exposure to environmental toxics such as pesticides (Yuan et al., 2019). Exposure to organophosphate pesticides, in particular the widely-used chlorpyrifos (CPF), results in alterations to core microbial communities. Previous studies have reported gut dysbiosis after exposure to CPF in male rats (Condette et al., 2015; Fang et al., 2018) and male mice (Liang et al., 2019; Zhao et al., 2016) and with the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) (Joly et al., 2013; Réquilé et al., 2018; Reygner et al., 2016). A link between alterations in microbiota composition and higher gut permeability has also been observed after exposure to different doses of the pesticide (Liang et al., 2019; Zhao et al., 2016). The general population is exposed to CPF mainly through diet (Reiss et al., 2015), being the gut microbiota directly exposed to CPF. CPF elicits its toxicity by the irreversible inhibition of the acetylcholinesterase

(AChE) enzyme, and there is evidence that developing organisms are more sensitive to this effect than adults (Burke et al., 2017; Moser, 1998). This is of particular relevance, since several studies have reported the existence of a crucial window during development when microbiota alterations can cause important long-lasting effects (Borre et al., 2014; Cox et al., 2014; Torow and Hornef, 2017).

Gut microbiota composition is also shaped by individual factors such as the genetic profile of the host (Bonder et al., 2016; Turpin et al., 2016), which can interact with environmental factors. A large study including monozygotic and dizygotic twins revealed that the relative abundance of certain microbiota elements is influenced by the genetic background of the subject (Goodrich et al., 2014). Tran et al. (2019) studied the influence of the different isoforms of apolipoprotein E (apoE) – a protein involved in lipid transport and homeostasis – on the composition of microbiota and its associated metabolites in both humans and transgenic mice. The results revealed the influence of the *APOE* genotype on the relative abundance of several bacteria taxa, which may in turn explain the differences observed in the associated metabolite levels (Tran et al., 2019). In humans, three isoforms – apoE2, apoE3 and apoE4 – which are functionally different, are mainly found, with apoE4 being a well-established risk factor for the development of dementia and Alzheimer's Disease (Hersi et al., 2017). Recent studies using the targeted replacement (TR) mouse model expressing the different isoforms of apoE (apoE-TR) showed different vulnerabilities to CPF exposure depending on *APOE* genetic background, including neurobehavioral (Basaure et al., 2019; Guardia-Escote et al., 2018) and metabolic disorders (Peris-Sampedro et al., 2015a, 2015b, 2018).

Gut microbiota send signals to the CNS via changes in immune reactivity, gut and serum metabolites and vagal nerve sensory innervation (Vuong et al., 2017). The short-chain fatty acids (SCFAs), which are produced by some microorganisms by the fermentation of non-digestible carbohydrates in the gut (Morrison and Preston, 2016) are key signaling factors of the gut-brain communication (Dalile et al., 2019). Different functions have been attributed to

SCFAs, for instance that they serve as an energy substrate for the gut cells, contributing to colonic epithelial integrity (Guilloteau et al., 2010; Morrison and Preston, 2016). Moreover, they can be transported via the portal vein, act as energy substrate or signaling molecules and influence the host metabolism in various tissues (De Vadder et al., 2014). Finally, a low percentage of SCFAs can cross the blood-brain barrier and enter the brain (Frost et al., 2014). A few authors have reported that levels of SCFA in the brain are low compared to those in the gut (Dalile et al., 2019). However, a correlation has been observed between the amount of some SCFAs in the gut and the brain, including butyrate, which may be involved in the modulation of several cerebral functions (Liu et al., 2015; Sun et al., 2016). In addition of being a source of energy for several cells, butyrate exerts its effects by acting as an inhibitor of histone deacetylases (HDACs) contributing in this way to the increase in gene expression in the host (Tan et al., 2014). This latter function can be of special relevance in developing organisms.

The aim of this investigation is to assess the effects on the composition of the gut microbiota of the most common apoE isoforms and those of postnatal exposure to the pesticide CPF. How they modulate the levels of SCFA in the brain at 15 days of age has also been investigated. To the best of our knowledge, this is the first study in which the gut microbiota and SCFA composition in the brain - at such a young age - in mice postnatally exposed to CPF has been assessed.

2. Material and methods

2.1. Animals and care

Male apoE-TR mice and C57BL/6 mice were used. Mice homozygous for the *ε3* and *ε4* allele were purchased from Taconic (Taconic Europe, Lille Skensved, Denmark). The apoE-TR model has its murine apoE gene replaced by the different alleles of human apoE (Sullivan et al., 1997). The C57BL/6 mice were obtained from Charles River (Charles River, Barcelona, Spain). Mice of the same genotype were mated during a one-week period, after which the females' body

weight was monitored. Pregnant females were kept in individual cages and the day of delivery was considered postnatal day (PND) 0. The animal room was maintained under standard conditions (temperature 22 ± 2 °C and relative humidity 50 ± 10%) with a 12h light/dark automatic light cycle. All the mice were allowed free access to water and fed a normal chow diet (Panlab, Barcelona, Spain). The use of animals and the experimental protocol were approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Spain) and were conducted in accordance with the Spanish Royal Decree 53/2013 on the protection of experimental animals, and the European Communities Council Directive (2010/63/EU).

2.2. Chemicals and treatment

CPF [0,0-diethyl O-(3,5,6-trichloropyridin-2-yl) phosphorothioate], 99.5% purity (Sigma-Aldrich Co. LLC., Madrid, Spain) was orally administered to the CPF-treated groups during PND 10-15. The compound was adjusted to administer 1 mg/kg in 1 μL/g of body weight. The control groups were given corn oil as the vehicle during the same period. A total of 36 male mice were divided into six experimental groups (n=6 animals/group).

2.3. Sacrifice and sampling

Biological samples were collected at PND 15, four hours after the last dose was administered. Animals were deeply anesthetized with isoflurane before being euthanized by decapitation. Gut and brain samples were collected and immediately stored at -80ºC.

2.4. Bacterial DNA extraction

Bacterial DNA was purified from the entire gut content obtained from 15-day-old mice using the PureLink™ Microbiome DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The DNA concentration in each sample was assessed by fluorometric quantification.

2.5. 16S metagenomics

The 16S metagenomics study was carried out in an external laboratory (Biomedica Molecular Medicine, Madrid, Spain). The specific region of the bacterial 16S rDNA gene V3+V4 was amplified by PCR with the conditions adapted to the DNA input in each sample. After marking, barcoding and quantifying, the resulting DNA was pooled and sequenced. Sequencing was performed using Illumina® MiSeq technology in order to obtain an approximate mean of 100,000 reads per sample. After the quality filter and barcode separation, the processing of the sequence reads was performed by Illumina 16S Metagenomics, version 1.0.1.0. Taxonomic assignation was performed in accordance with the Greengenes database. Each 16S rRNA sequence was assigned at different taxonomic levels, such as phylum, genus and species.

2.6. Analysis of short-chain fatty acids

SCFA determination was carried out in an external laboratory (Centre for Omic Sciences (COS), Reus, Spain). Whole brain samples were homogenized and lipids were extracted from a total volume of 100 mg of the homogenized tissue. Chromatographic separation was performed with a gradient elution using a Kinetex polar C18, 2.6 μm 2.1 x 100 mm analytical column (Phenomenex, Torrance, CA, USA). Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, 3-methylvaleric acid, 4-methylvaleric acid and hexanoic acid were quantified in all samples. Metabolite concentration was normalized using tissue weight and expressed as pmol/mg or pmol/g tissue.

2.7. Statistical analysis

Data were analyzed using SPSS 25.0 software (IBM Corp, Chicago, USA). Principal component analysis (PCA) was performed in order to observe intrinsic clusters in the gut microbiota data. A two-way analysis of variance (ANOVA) was used to study the relative abundance of the different microbiota components and the concentration of SCFAs. A one-way ANOVA and *post-hoc* Tukey's tests of variance were used to analyze differences between groups. Pearson correlations were used to investigate associations between microbiota

composition and SCFAs levels. Variance homogeneity was assessed by a Levene test. The results are represented as mean values ± S.E.M. Statistical significance was set at $p<0.05$.

3. Results

3.1. Gut microbiome

The relative abundance of each phylum, genus and species was calculated by dividing each read count by the total read count for each sample. There were no differences between groups regarding diversity, assessed using the Shannon diversity index (Kim et al., 2017) (data not shown).

3.1.1. The phylum *Verrucomicrobia* was highly present in apoE4 mice, while postnatal exposure to CPF tended to equal the levels between genotypes

The gut bacteria assigned at phylum level are shown in Figure 1, with only the most abundant phyla represented (i.e. subjects with a >1% relative abundance). It can be seen that the predominant phylum is *Bacteroidetes* (48.05-69.39%), followed by *Firmicutes* (18.01-26.69%) and *Proteobacteria* (7.03-11.50%). Interestingly, *Verrucomicrobia* was highly present (26.34%) only in apoE4 mice. Indeed, a two-way ANOVA (genotype x treatment) indicated significant effects of the genotype on this phylum [$F(2,30)=9.490$, $p=0.001$] and a tendency towards significant effects of the treatment [$F(1,30)=4.091$, $p=0.052$] and the interaction between genotype and treatment [$F(2,30)=3.295$, $p=0.051$]. In order to further study these differences, we performed a one-way ANOVA (group) (Fig. 2). *Post-hoc* analysis revealed that the control-apoE4 group presented significantly higher levels of *Verrucomicrobia*, suggesting a basal difference between apoE4 and the other genotypes, which is attenuated by postnatal exposure to CPF (Fig. 2A).

Further study of the less abundant phyla showed significant differences. For example, the phylum *Nitrospirae* showed a significant influence of the genotype [F(2,30)=6.709, p=0.004] and the treatment [F(1,30)=7.268, p=0.011], and a tendency towards interaction between genotype and treatment [F(2,30)=3.253, p=0.053] (Fig. 2B). The phylum *Thermi* displayed significant effects of the genotype [F(2,30)=4.280, p=0.023] (Fig. 2C), while the phyla *Thermotogae* and *Chrysiogenetes* showed effects of the treatment ([F(1,30)=7.401, p=0.011] and [F(1,30)=8.155, p=0.008] respectively) (Fig. 2D and 2E). Finally, the phylum *Planctomycetes* presented an effect of the genotype [F(2,30)=9.103, p=0.001], the treatment [F(1,30)=6.929, p=0.013] and the interaction between genotype and treatment [F(2,30)=5.783, p=0.008] (Fig. 2F). Further *post-hoc* analysis revealed that *Nitrospirae* and *Planctomycetes* showed the same pattern of differences between groups as that previously described for *Verrucomicrobia*, with the control apoE4 being the group showing the highest levels of the microorganism compared to the other groups.

3.1.2. The *APOE4* genotype differs from the other genotypes in the *Akkermansia*, *Luteolibacter* and *Rubritalea* genera, with the *APOE4* genotype being the only one affected by CPF exposure.

We performed a principal component analysis (PCA) on the most abundant gut bacteria (i.e. subjects presenting >1% relative abundance) assigned at genus level (Fig. 3). PCA was used to explore the different clustering patterns of the genera studied, with the variance explained by the principal components (PC). PC 1 explained 16.54% of the variance, while PC 2 explained 13.78% and PC 3 explained 8.88%. We found some genera that were clustered together. Therefore, to study them in a greater detail we performed a two-way ANOVA (genotype x sex) (Supplementary Table 1). Significant effects of the genotype [F(2,30)=9.748, p=0.001], the treatment [F(1,30)=4.240, p=0.048] and interaction between genotype and treatment [F(2,30)=3.425, p=0.046] on the genus *Akkermansia* were observed (Fig. 4A). The effects of genotype were also noticed in *Escherichia* [F(2,30)=4.235, p=0.024] (Fig. 4B), *Helicobacter* [F(2,30)=3.611, p=0.039] (Fig. 4C), *Parapedobacter* [F(2,30)=3.745, p=0.035] (Fig. 4D),

Enterococcus [F(2,30)=3.452, p=0.045] (Fig. 4E), *Serratia* [F(2,30)=4.512, p=0.019] (Fig. 4F), *Luteolibacter* [F(2,30)=9.439, p=0.001] (Fig. 4G) and *Rubritalea* [F(2,30)=4.008, p=0.001] (Fig. 4H). Furthermore, *Helicobacter* and *Streptococcus* (Fig. 4I) also presented significant interaction between genotype and treatment ([F(2,30)=5.121, p=0.012] and [F(2,30)=5.289, p=0.011] respectively). In order to further study these differences, we performed a one-way ANOVA (group). *Post-hoc* analysis revealed that the control-apoE4 group presented an enrichment of *Akkermansia, Luteolibacter* and *Rubritalea* compared to the other groups, whereas the CPF-treated apoE4 mice presented an enrichment of *Helicobacter*. These results suggest that *APOE4* carriers differ from the other genotypes, being the *APOE4* genotype the most susceptible to the effects of the pesticide on gut microbiota composition.

3.1.3. The *APOE4* genotype differs from the other genotypes in the species *Akkermansia muciniphila*.

In order to gain further insight into the effects already observed, we studied the species in those genera showing significant differences. Those whose relative abundance was significantly affected by the *APOE* genotype and CPF exposure are shown in Supplementary Table 2. The results point to the importance of *Akkermansia Muciniphila.* A two-way ANOVA (genotype x treatment) showed a significant effect of the genotype [F(2,30)=9.748, p=0.001], the treatment [F(1,30)=4.240, p=0.048] and the interaction between genotype and treatment [F(2,30)=3.425, p=0.046]. Post-hoc analysis revealed that the apoE4 control group was significantly different from the other groups, accounting for more than 24-fold the amount found in the C57BL/6 control group. ApoE4 was again the group showing the highest levels, while postnatal CPF exposure considerably diminished the presence of this species. Interestingly, a similar effect was also observed at a considerably lower percentage in different species belonging to the same *Verrucomicrobia* phylum (i.e. *Luteolibacter algae, Prosthecobacter fluviatilis* and *Coraliomargarita akajimensis*).

3.2. The *APOE3* genotype and CPF treatment increased the levels of certain SCFAs.

We studied the concentrations of different SCFAs in the brain (Fig. 5). Differences between genotypes were found in three of the most abundant SCFAs: acetic acid [$F(2,30)=11.069$, $p<0.001$] (Fig. 5A), propionic acid [$F(2,30)=3.399$, $p=0.047$] (Fig. 5B) and butyric acid [$F(2,30)=33.692$, $p<0.001$] (Fig. 5B). The latter also presented an interaction between genotype and treatment [$F(2,30)=4.044$, $p=0.028$]. In all cases, the apoE3-TR mice showed higher concentrations compared to the other groups. Differences between treatments (Fig. 6) were observed in isovaleric acid [$F(1,30)=1.204$, $p=0.044$] (Fig. 6A) and 4-methylvaleric acid [$F(1,30)=4.432$, $p=0.044$] (Fig. 6B). In both cases, animals in the CPF-treated group presented higher concentrations than those in the control group. The remaining SCFAs studied (isobutyric acid, 3-methylvaleric acid, valeric acid and hexanoic acid) did not show significant differences between groups.

3.3. Certain SCFAs presented mild correlations with the relative abundance of several intestinal species.

In order to identify any correlations between the relative abundance of the microbiota species of interest and SCFA concentrations in the brain, we performed a Pearson's correlation (Table 1). Mild correlations were noticed between isobutyric acid and the relative abundance of *Coraliomargarita akajimensis*, *Selenomonas infelix* and *Candidatus Scalindua brodae*. Furthermore, a relative abundance of *Methylobacillus glycogenes* positively correlated with the concentration of valeric acid, 3-methylvaleric acid and hexanoic acid, suggesting that SCFA production in the gut might influence the brain levels.

4. - Discussion

The aim of the present study was to assess the effects of the *APOE* genotype and postnatal exposure to the pesticide CPF on gut microbiota composition and SCFA concentrations in the

brain. The relative abundance of the microbiota at PND 15, four hours after exposure to CPF, was studied at different taxonomic levels: phylum, genus and species. The results revealed not only changes induced by the pesticide but also important changes depending on the host's *APOE* genetic background – and the interaction between the two – implying that the microbiota can be modulated by both the host's genetics and environmental factors. SCFA concentrations in the brain also proved to be susceptible to the effects of the genotype and the treatment, presenting a mild correlation with the changes observed in microbiota composition. Infant microbiota is influenced by factors such as the delivery process, the maternal environment and milk composition (Borre et al., 2014). Differences between lactation and adulthood periods have been reported (Bäckhed et al., 2015; Palmer et al., 2007), but little is known about milk composition at early stages and its influence on brain development.

In the present study, microbiota composition differed between genotypes at different taxonomic levels, with the *APOE4* genotype being the one diverging from the others in the vast majority of cases. Since no differences in diversity as assessed using the Shannon index were observed, it can be stated that apoE4 differed from the other genotypes in the proportion of some microorganisms. A recent study including both human and apoE3- and apoE4-TR mice at two different ages during adulthood also found that the *APOE* genotype influenced the relative abundance of microbiota (Tran et al., 2019). Specifically, they observed differences in the most abundant phyla: apoE4-TR mice presented a higher amount of *Deferribacteres* and a lower relative abundance of *Candidatus Saccharibacteria* and *Proteobacteria* compared to apoE3-TR mice (Tran et al., 2019). In the current study, no differences in the same phyla were found, but we did observe a higher relative abundance of *Verrucomicrobia*, *Nitrospirae* and *Planctomycetes* in apoE4-TR mice in comparison with the rest of genotypes. However, we found genotype differences in genera belonging to the phylum *Proteobacteria*: *Helicobacter*, *Escherichia*, *Enterobacter* and *Serratia*, among others. Both studies used the same animal model, but determinations in the previous study (Tran et al., 2019) used fecal samples and adult

mice, whereas the determinations in our study were performed on total gut content and lactating mice. Microbiota composition can change during the postnatal period (Pantoja-Feliciano et al., 2013), which makes it difficult to compare results from different developmental stages. Tran et al. (2019) also found differences between genotypes in human samples, since E2/E3 presented higher amounts of *Firmicutes* than E3/E4 and E4/E4, which were found neither in adult mice (Tran et al., 2019) nor in our lactating mice. However, it must be taken into account that the data in human studies may be compromised by diet variations, infections or antibiotic use, as well as environmental factors. In the present case, given that the determinations were conducted during the lactation period, we cannot rule out that any differences in the nutritional composition of maternal milk or differences in the nutrient conversion between genotypes may be contributing to the observed differences. In fact, breast milk plays an important role in establishing a healthy gut microbiota (Walker and Iyengar, 2015), and differences in its composition could enhance the proliferation of different microorganisms. We have recently found different maturation patterns for apoE3- and apoE4-TR mice (Basaure et al., 2018). Since the presence of *Akkermansia muciniphila* increases with age (Collado et al., 2007; Derrien et al., 2008), an earlier maturation of apoE4 could also be underlying its higher relative abundance compared to the other genotypes.

In recent years, the impact of CPF on the gut microbiota has been thoroughly explored with all studies agreeing on the potential effect of CPF to induce dysbiosis. In the present investigation we assessed the effects of postnatal exposure to CPF, in contrast to most studies, which have mainly focused only on adult exposure. Nevertheless, Condette et al. (2015) studied in male rats the effects of a gestational and developmental exposure to CPF until 60 days of age. It was noticed that CPF exposure during this period induced dysbiosis, and lower levels of microorganisms related to healthy phenotypes (i.e. *Lactobacillus* spp) and higher levels of microorganisms related to unhealthy phenotypes (i.e. *Enterococcus* spp, *Clostridium* spp and *Staphylococcus* spp) at PND 21 and PND 60 were found in different parts of the intestine

(Condette et al., 2015). Furthermore, they also found detrimental effects on the normal development of the intestine (Condette et al., 2015) and compromised integrity of the epithelial barrier function in rats (Joly Condette et al., 2014). Exposure during adulthood has also revealed detrimental effects in gut permeability and alterations in the relative abundance of key microorganisms, with a decrease in the relative abundance in the phylum *Firmicutes*, and an increase in *Bacteroidetes* in the CPF-exposed mice (Zhao et al., 2016). Despite the fact that we did not observe any differences in those phyla, we found some changes in the same direction after CPF exposure. More specifically, exposure to CPF decreased the relative abundance of *Streptococcus* in C57BL/6 mice but increased *Rhodothermus* in apoE4-TR mice. It is worth noting that CPF can have a direct effect on the relative abundance of some microorganisms. Overall, these results corroborate the disturbing effects of CPF on microbiota composition and identify new microorganisms affected by the genotype and CPF at very early ages.

The three most abundant phyla (*Bacteroidetes*, *Firmicutes* and *Proteobacteria)* presented no differences between groups, but differences were found in *Verrucomicrobia* and in some of the less abundant phyla. Interestingly, when we further studied the genus and species belonging to the *Verrucomicrobia* phylum, we observed the same pattern. The species *Akkermansia muciniphila*, *Luteolibacter algae*, *Phrosthecobacter fluvialis*, and *Coraliomargarita akajimensis* all presented the same differences between groups, i.e. the control apoE4 presented a significantly higher abundance than the other groups. This implies a basal difference between genotypes, but also a significant effect of the treatment only in the *APOE4* genotype, as postnatal exposure to CPF decreased the relative abundance of this group of microorganisms. In fact CPF exposure would decrease the existing variability between genotypes, making *APOE4* more comparable to the other genotypes. Of special relevance is the *A. muciniphila* species, a mucin-degrading bacterium (Derrien et al., 2004), which is present from early life in infants (Collado et al., 2007), and it is positively correlated with a healthier metabolic status (Dao et al., 2016) and negatively correlated with obesity and diabetes (Everard et al., 2013). Several studies

suggest *A. muciniphilla* as a new treatment for obesity and diabetes (Cani and de Vos, 2017; Zhang et al., 2019). Indeed, it has been demonstrated that administration of viable *A. muciniphilla* would ameliorate the metabolic profile, improve the gut barrier function and reduce inflammation, and reverse diet-induced metabolic disorders in mice (Everard et al., 2013). Differences between the metabolic profiles of *APOE* genotypes have been observed before (Huebbe et al., 2015). The *APOE3* genotype has been associated with greater vulnerability to the development of obesity, while *APOE4* has normally been associated with a leaner phenotype (Huebbe et al., 2015). We hypothesize that this different vulnerability to obesity and diabetes of apoE3- and apoE4-TR mice may in part be influenced by the presence of *A. muciniphilla* since early life, conditioning the long-term metabolic health of the subject.

In this sense, in a previous study, apoE3-TR male mice presented more signs of insulin resistance, obesity and metabolic diseases after dietary exposure to CPF during adulthood compared to their peers: apoE4-TR and C57BL/6 mice (Peris-Sampedro et al., 2015a, 2015b). It is known that CPF can impact the metabolism and increase the incidence of several metabolic conditions (Acker and Nogueira, 2012; Elsharkawy et al., 2013). Studies including a dietary challenge with a high-fat diet have suggested that CPF may alter microbiota composition and be associated with an obese phenotype in a diet-specific manner (Fang et al., 2018). In fact Liang et al. (2019) hypothesize that these effects may be the final outcome of disruptions in the gut microbiota composition that alter gut permeability and consequently increase the release of LPS to the body, resulting in inflammation. *A. muciniphilla* normally resides in the mucus layer of the intestinal epithelium (Ouwerkerk et al., 2016). Thus, we can speculate that alterations in this species could compromise its integrity. It has been reported that low-grade inflammation after CPF exposure enhanced body weight increase in mice, impaired glucose homeostasis and induced insulin resistance (Liang et al., 2019). Indeed, a higher presence of *A. muciniphilla* has been observed in children with autism (De Angelis et al., 2013) and in studies with the BTBR inbred mouse model, which presents the full spectrum of symptoms associated with autism

spectrum disorder (Needham et al., 2018). Higher levels of *A. muciniphilla* have also been detected in Parkinson disease patients (Bedarf et al., 2017), suggesting the potential implication of *A. muciniphilla* in altered neurobiological processes.

In the recent years important attention has been paid to the SCFA produced by microorganisms in the gut and its signaling effects. Previous studies suggest an important role of SCFAs in the cerebral function. For instance, SCFAs can carry out an important function in communication between gut and microglia. Moreover, acetate acts as an important energy source for astrocytes (Deelchand et al., 2009) while butyrate can play a neuroprotective role (Sun et al., 2016). However, physiological levels of SCFAs in the brain are largely unknown, especially during developmental period. The current results reveal that SCFA composition is modulated by the *APOE* genotype and also by postnatal exposure to CPF in the brain of lactating mice. In the present study, the levels of acetic acid, butyric acid and propionic acid, which are considered to be the three most common SCFAs (Cummings et al., 1987), were influenced by the genotype. Specifically, apoE3 presented higher concentrations than the other genotypes. We found that levels of butyrate in brains of our mice are about five to ten times higher than those previously found in mature mice (Liu et al., 2015; Sun et al., 2016). Since butyrate serve several functions such as being an energy substrate for cells (Donohoe et al., 2011; Maslowski, 2019); signaling via interaction with G-protein-coupled receptors and the inhibition of histone deacetylases stimulating gene expression (Tan et al., 2014), the current results provide new insights to be explored as a source of early variations related to the *APOE* genotype. We hypothesize that these differences may already be influencing the cerebral function, conferring higher neuroprotection to apoE3 in contrast to apoE4. This would be in accordance with the higher susceptibility of apoE4 to develop cognitive impairments and AD later in life. An important question is, which are the sources of SCFAs in brain? It is known that only a small part of the SCFAs produced would pass through the blood-brain barrier and enter the brain (Frost et al., 2014). Another important source of SCFAs is the diet being dairy products an important source

for butyrate (Stilling et al., 2016). It is also worth considering that SCFAs in the form of acetate, butyrate and propionate can be derived from endogenous sources such as fatty acid oxidation and glucose metabolism in a lower level (Bourassa et al., 2016; Pouteau et al., 2003; Wolever et al., 1997). In the present case, it must be taken into account that the composition of mother's milk, as the only source of diet in lactating mice, can be an important factor contributing to the differences found. Albeit speculative, if differences between maternal milk composition exist, early interventions could be planned.

We observed an effect of CPF on the cerebral concentrations of isovaleric acid and 4-methylvaleric acid, increasing the concentration in all genotypes except for apoE4, which remained unaltered. As mentioned above, CPF can alter gut permeability, and therefore it can be hypothesized that it may favor the release of less abundant SCFAs into the blood, which may then reach the brain at higher concentrations. A link between higher levels of isovaleric acid in stool and depression has been reported in human patients (Szczesniak et al., 2016), correlated with higher levels of certain microorganisms previously linked to this condition. This study suggests that isovaleric acid may be inducing this effect through the inhibition of Na^+, K^+-ATPase (Szczesniak et al., 2016), and thus it can be conjectured that increased levels could cause important damage to the developing brain. Zhao et al. (2016) have reported changes in urine metabolic profiles after exposure to CPF, which are strongly correlated with changes in SCFA-producing bacteria. These metabolites, which included SCFAs such as isobutyrate and valerate, showed changes after CPF exposure (Zhao et al., 2016). It is worth noting that we observed a general effect of the treatment in several metabolites in the brain, but apoE4-TR mice remained unaltered. This would suggest that *APOE3* carriers are more sensitive to the effects of CPF, while *APOE4* carriers would not be affected by the pesticide in this aspect. This finding has important implications because of the $\mathcal{E}3$ allele is the most conserved in the human population worldwide, reaching a frequency of 73.3% (SD=12.1). In contrast, the $\mathcal{E}4$ allele is only present in 14.5% (SD=8.5) (Eisenberg et al., 2010), indicating that a large number of

people could be susceptible to these toxic-induced changes in their SCFA levels. Insofar as SCFA can influence the regulation of feeding behavior, these differences are along similar lines to the metabolic differences between apoE3 and apoE4, which have been discussed above. Even though the direct relationship between SCFAs in the gut and microbiota composition is incontestable, information on cerebral SCFAs is scarce. It has been previously reported that supplementation with the butyrate-producer specie *Clostridium butyricum* would have a direct effect by increasing butyrate levels in the brain and by ameliorating cognitive impairments in a mouse model of vascular dementia (Liu et al., 2015). However, in the present case we did not find a strong correlation between the two factors.

The results of this study were obtained 4 h after exposure to low doses of CPF from PND 10 to PND 15. This would raise the question of whether the results obtained represent a permanent effect or only an acute response that could disappear over time. Changes in the gut microbiota due to environmental toxics during this critical development period have shown to create permanent impairments that can condition the whole life of the subject (Torow and Hornef, 2017). However, further investigation is required to assess whether this also happens with CPF, and to study the evolution over time of the differences between genotypes at such an early age. Last but not least, as most of these studies with mouse models, our results are based only on males. However, considering that we recently observed sexual-dimorphic differences and vulnerabilities to toxic exposures (Basaure et al., 2018; Guardia-Escote et al., 2018), we believe that we might be missing important information by leaving the females out of these studies. Consequently, we propose a long-term follow-up study including both sexes, with a strict control of all possible parameters influencing the gut microbiota.

In summary, the current results provide information about the composition of gut microbiota in early life and its modulation by the *APOE* genetic background and postnatal exposure to CPF.

We also included an assessment of SCFA levels in the brain and studied the potential relationship between *APOE* and CPF. Basal differences between genotypes were observed, and CPF showed its capacity to induce gut dysbiosis. *A. muciniphilla* emerged as a potential modulatory element providing an explanation for previously-observed metabolic and cognitive differences between groups, especially related to *APOE4* genotype. Genetic and environmental effects on SCFA composition were also found, with potential implications for cognitive functioning. Finally, these findings raise a number of intriguing questions regarding the nature and extent of the involvement of the gut microbiota and its metabolites in different aspects underlying health and disease. At the same time, they show the scope of postnatal exposure to low doses of pesticides such as CPF and corroborate the influence of the *APOE* genotype.

Declaration of Interests

The authors declare no competing interests.

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Figure legends

Figure 1| Relative abundance of the most abundant phyla (i.e. subjects with a >1% relative abundance). Different letters represent significant differences between groups at $p<0.05$.

Figure 2| Relative abundance of phyla presenting significant differences between groups. (A) *Verrucomicrobia*, (B) *Nitrospirae*, (C) *Thermi*, (D) *Thermotogae*, (E) *Chrysiogenetes* and (F) *Planctomycetes*. Different letters represent significant differences between groups at $p<0.05$.

Figure 3| Principal component analysis (PCA) with the relative abundance of the most abundant genera (i.e. subjects with a >1% relative abundance). PC1 included *Akkermansia*, *Escherichia*, *Helicobacter*, *Pedobacter*, *Parapedobacter*, *Serratia*, *Luteolibacter*, *Rubritalea* and *Bilophila*. PC2 included *Parabacteroides*, *Lactobacillus*, *Aggregatibacter*, *Ruminococcus*, *Olivibacter*, *Anaeroplasma*, *Slackia*, *Mucispirillum* and *Proteus*. PC3 included *Blautia*, *Bacteroides*, *Oscispirilla*, *Enterococcus* and *Acetobacterium*.

Figure 4| Relative abundance of the most abundant genera (i.e. subjects with a >1% relative abundance) showing significant differences between groups. (A) *Akkermansia*, (B) *Escherichia*, (C) *Helicobacter*, (D) *Parapedobacter*, (E) *Enterococcus*, (F) *Serratia*, (G) *Luteolibacter*, (H) *Rubritalea* and (I) *Streptococcus*. Different letters represent significant differences at $p<0.05$.

Figure 5| Brain concentrations of SCFAs presenting significant differences between genotypes. (A) Acetic acid, (B) Propionic acid and (C) Butyric acid. Different letters represent significant differences at $p<0.05$.

Figure 6| Brain concentrations of SCFAs presenting significant effects of the postnatal treatment. (A) Isovaleric acid and (B) 4-Methylvaleric acid. Different letters represent significant differences at $p<0.05$.

Table

Table 1. Correlation between gut microbiota species and SCFA composition

Specie	SCFA	Correlation	p-value
Coraliomargarita akajimensis	IBA	r =0.532	p=0.001
Selenomonas infelix	IBA	r =0.554	p<0.001
Candidatus Scalindua brodae	IBA	r =0.544	p<0.001
Methylobacillus glycogenes	VA	r =0.523	p=0.001
Methylobacillus glycogenes	3-MVA	r =0.810	p<0.001
Methylobacillus glycogenes	HA	r =0.560	p<0.001

Figure 1

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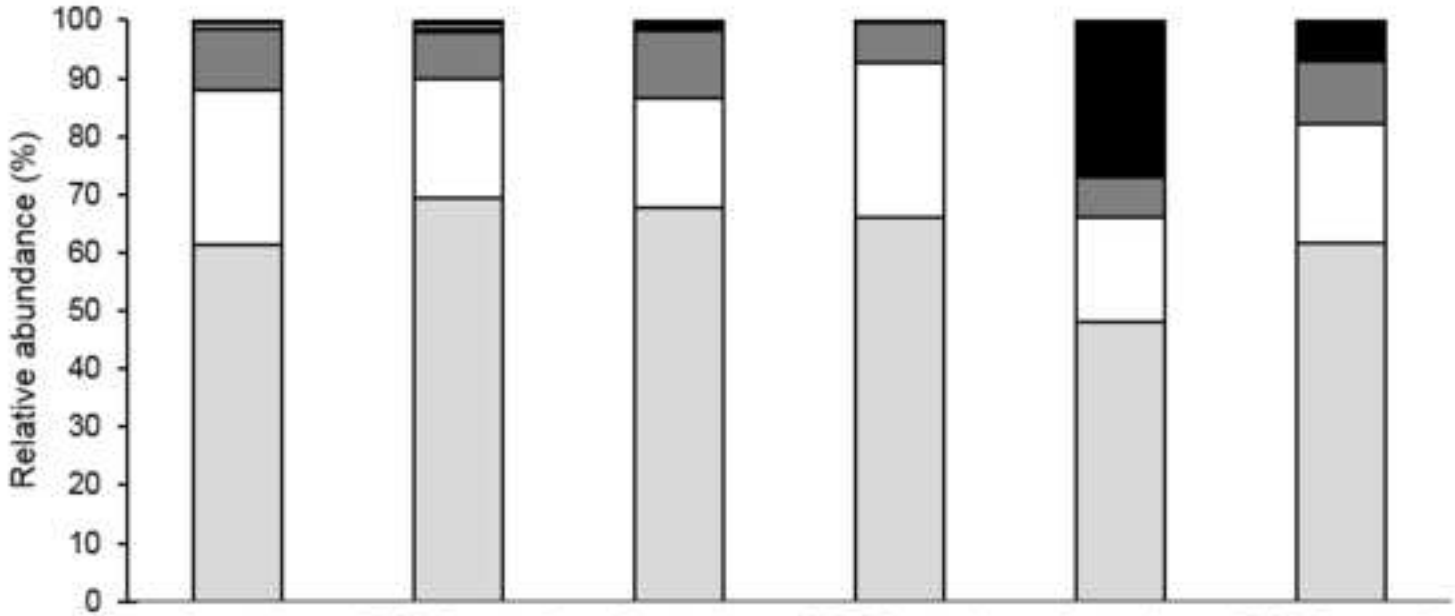

	Control C57BL/6	CPF-Treated C57BL/6	Control apoE3	CPF-Treated apoE3	Control apoE4	CPF-Treated apoE4
Bacteroidetes	61.38±6.07	69.39±3.08	67.85±4.54	65.94±7.47	48.05±5.93	61.63±5.98
Firmicutes	26.55±6.60	20.60±4.26	18.75±3.97	26.69±8.62	18.01±2.92	20.38±3.63
Proteobacteria	10.65±2.56	7.96±3.36	11.50±2.24	7.03±3.46	7.03±1.17	10.84±1.25
Verrucomicrobia	0.02±0.003[a]	0.01±0.004[a]	1.49±1.48[a]	0.02±0.005[a]	26.34±9.12[b]	6.65±4.93[a]
Cyanobacteria	0.99±0.06	0.39±0.19	0.23±0.05	0.15±0.02	0.16±0.05	0.18±0.03
Tenericutes	0.20±0.10	0.98±0.97	0.06±0.02	0.08±0.05	0.05±0.02	0.08±0.05
Deferribacteres	0.01±0.00	0.46±0.29	0.002±0.00	0.001±0.00	0.11±0.07	0.12±0.08
Other	0.21±0.09	0.21±0.05	0.10±0.01	0.09±0.01	0.24±0.07	0.12±0.01

Figure 2

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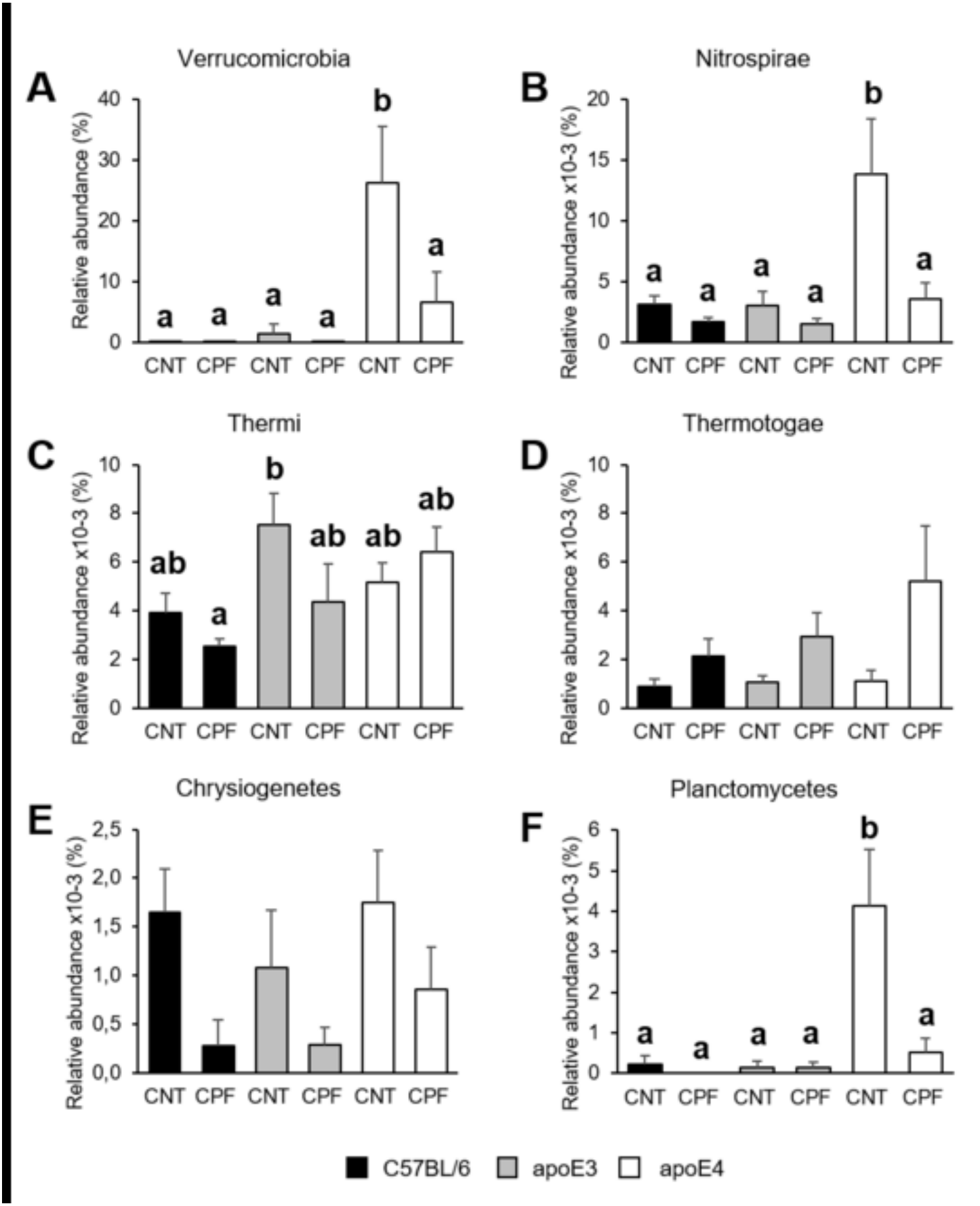

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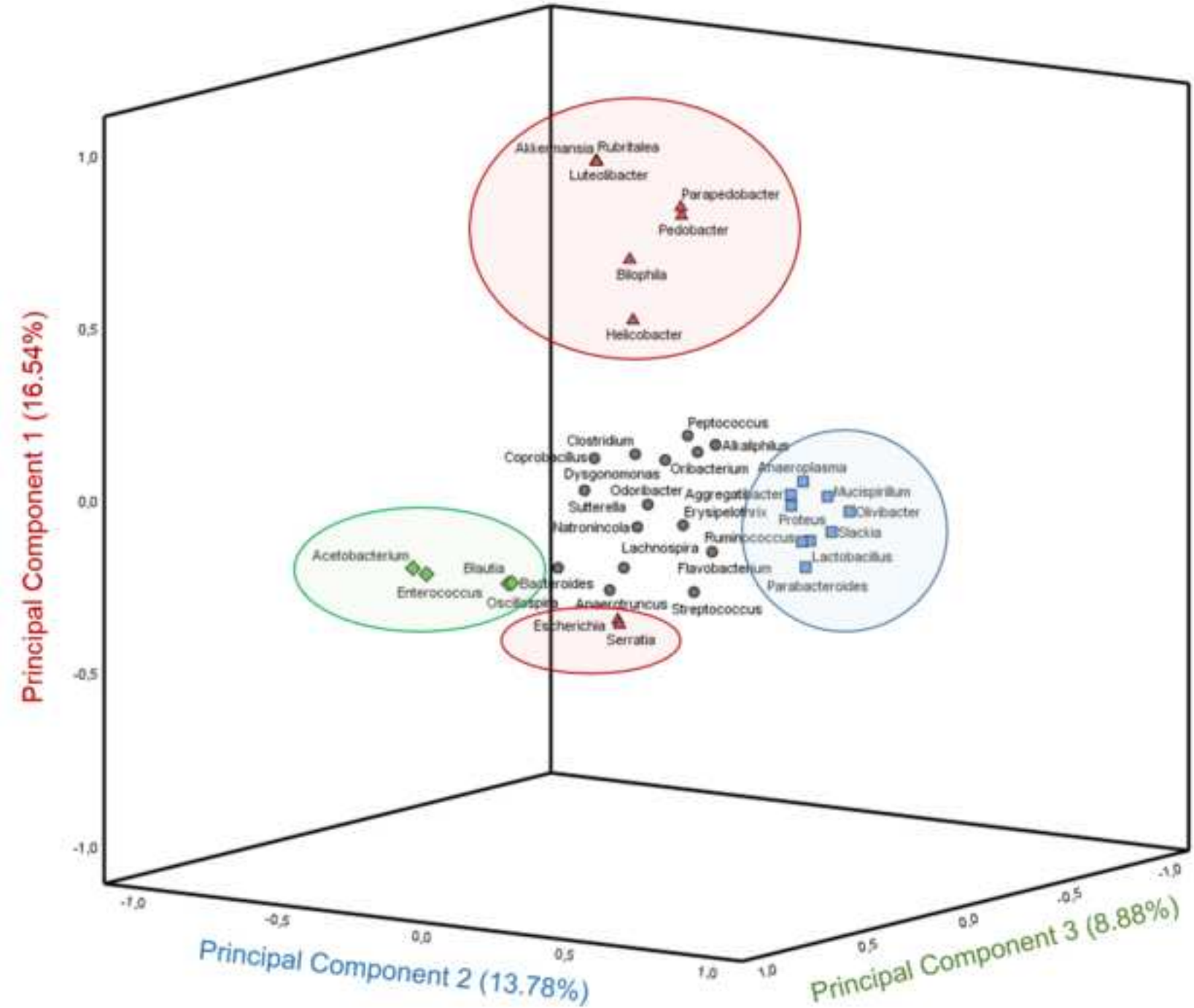

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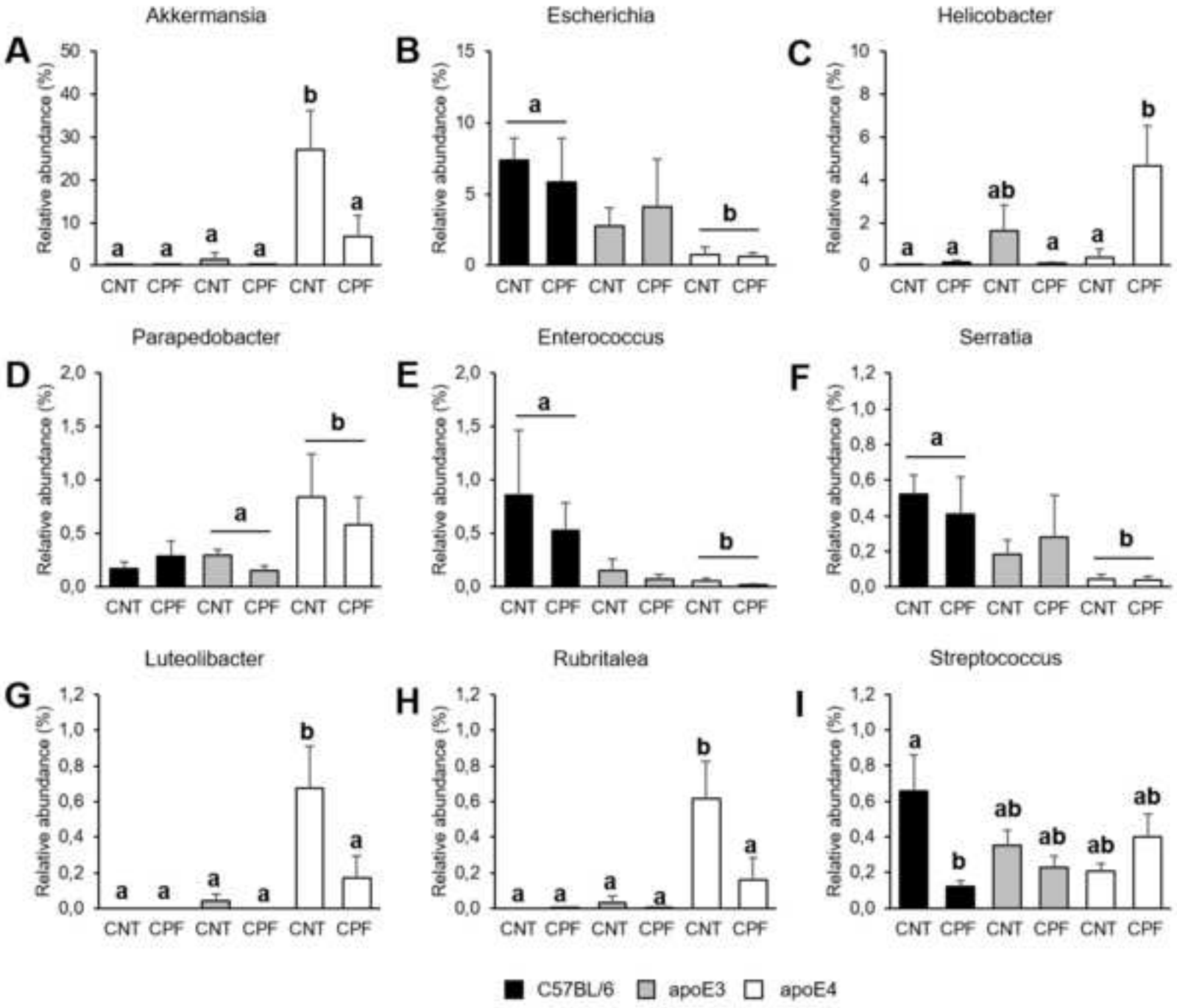

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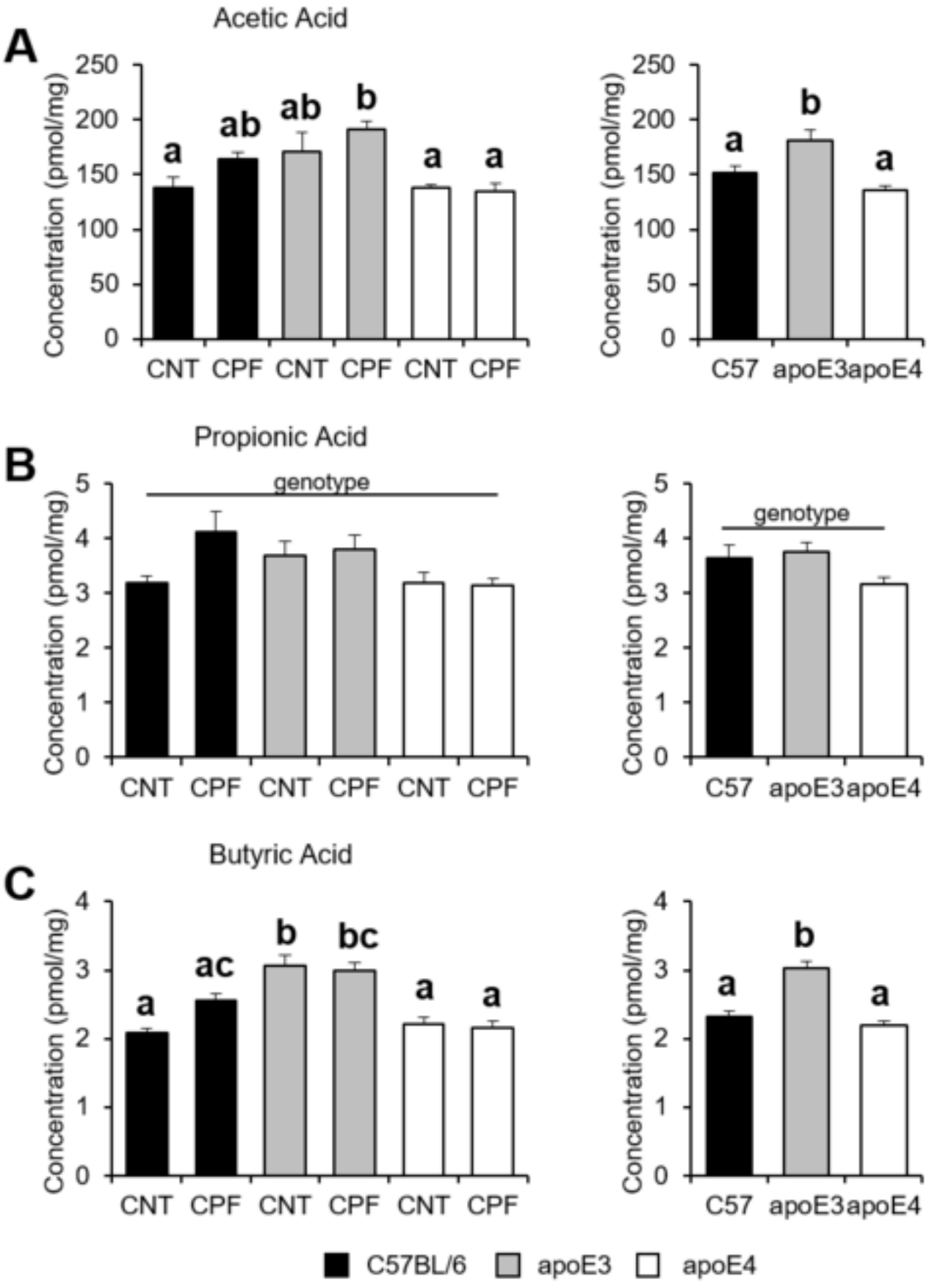

Figure 6

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