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Running title: BPA exposure exacerbates food allergy

Abstract

Allergic diseases are increasing worldwide, and their precise causes are not fully understood. However, this observation can be correlated with growing chemical pollution of the environment. Bisphenol A (BPA) alters the immune system, microbiota and barrier functions. Here, we studied the effect of oral BPA at levels equivalent to human exposure to understand the mechanisms of immunological, physiological and microbial action on food allergies. In a murine model of allergy, we evaluated the effect of direct oral exposure to BPA at 4 µg/kg bw/d corresponding to tolerable daily intake (TDI). We studied symptoms, intestinal physiology and humorall and cellular immune responses during food allergy. We explored the relationship between oral exposure to BPA and changes in the gut microenvironment. Markers of food allergy and intestinal permeability were increased following exposure to BPA. We also observed a modulated humorall and T-cell response with aggravation of food allergy inflammation. Moreover, BPA exposure induced gut dysbiosis and decreased microbial diversity induced by food allergy. Altogether, these results suggest that the 2015 European Food Safety Authority (EFSA) TDI should be reviewed to consider the immunotoxicity of BPA.

Keywords: Allergy, Bisphenol A, Wheat, Mouse model, T lymphocytes

Abbreviations used

BPA: Bisphenol A

DCs: Dendritic cells

ECHA: European Chemical Agency

EDCs: Endocrine-disrupting compounds

EFSA: European Food Safety Authority

EPA: Environmental Protection Agency

FA: Food allergy

IBD: Inflammatory bowel disease

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL: Interleukin

mLn: Mesenteric lymph node

NGs: Native gliadins

OVA: Ovalbumin

SEM: Standard error of the mean

T2D: Type 2 diabetes

TDI: Tolerable daily intake

US: United States

1. Introduction

Allergic diseases are increasing worldwide, with 20% to 30% of the population affected within industrialized countries(Pawankar et al. 2011). This observation can be attributed to the rapidly growing chemical pollution of the environment. Humans are constantly exposed to natural and synthetic chemical compounds. Among them, xenoestrogens, oestrogen-like compounds of exogenous origin that act as endocrine-disrupting compounds (EDCs), play an important role. Sources of xenoestrogens include chemicals used to harden plastics, such as bisphenol A (BPA)(Fenichel et al. 2013; Rochester 2013). The popularity of plastic products has led to the widespread use of BPA. Used in polycarbonate plastics and epoxy resins, BPA has been detected in children's toys, dental sealants, medical devices, internal coating of cans and food and beverage containers(Abraham and Chakraborty 2020; Lofroth et al. 2019). It easily penetrates food and beverages at elevated temperatures or due to damage caused at the time of packaging. Humans are exposed to this compound mainly through food products, and the absorption is particularly high in children. Therefore, BPA is ubiquitous and can be detected in blood and urine samples in 95% of the U.S. population, according to biomonitoring surveys by the U.S. Centers for Disease Control and Prevention(Calafat et al. 2005). Based on several studies, the United States Environmental Protection Agency (EPA) set a reference dose of 50 µg/kg bw/d for BPA. As a response to a refined risk assessment of BPA and its unwanted health effects, the European Food Safety Authority (EFSA) decreased the tolerable daily intake (TDI) from 50 µg/kg bw/d to 4 µg/kg bw/d in 2015 (EFSA journal, 2015). However, the EFSA also claimed that the average levels of BPA exposure are far below the TDI and that BPA does not increase the health risks for any population, including unborn children, infants and young people. In 2017, BPA

was listed as a substance of very high concern by the European Chemical Agency (ECHA).

BPA is known to exert harmful effects on human health, even at low concentrations below ng/ml body weight(Chen et al. 2016; Vandenberg et al. 2013). It can bind to oestrogen receptors, oestrogen-related receptors, aryl hydrocarbon receptors and peroxisome proliferator-activated receptors(Acconcia et al. 2015). Owing to its ability to interact with many types of receptors, BPA has a wide impact on immune system regulation(Xu et al. 2016). Regarding the impact of BPA on the immune/allergic system, an epidemiological study showed that prenatal exposure to BPA was associated with paediatric respiratory outcomes among boys but not girls(Buckley et al. 2018). The urine concentration of BPA is related to atopic dermatitis symptom aggravation in children(Kim et al. 2017a). In animal studies, Bauer *et al.* reported that prenatal and postnatal exposure to BPA enhanced allergic lung inflammation in female but not male offspring of mice(Bauer et al. 2012). These studies have focused on indirect effects, such as prenatal exposure. To analyse the direct impact of BPA on allergic responses, He *et al*. reported that repetitive oral exposure to BPA (1 mg per mouse 4 times) enhanced lung eosinophilia by promoting Th2-type immune responses in allergen-sensitized male mice(He et al. 2016). However, the effects of low-dose BPA are comparable to the level of human exposure, and the underlying mechanisms are poorly understood. Recently, Yanagisawa *et al.* showed that oral exposure to low-dose BPA (equivalent to a dose of 0.09 μg/kg/day) promoted airway hyperresponsiveness and mesenteric lymph node (mLN) activation in OVA-sensitized mice(Yanagisawa et al. 2019). In animals, previous studies from our laboratory highlighted a deleterious effect of BPA oral exposure on intestinal physiology. Indeed, we previously showed in studies that daily

gavage with BPA of rodent mothers from gestational Day 15 to pup weaning increased the risk of food intolerance in adulthood(Menard et al. 2014b). Similarly, BPA treatment impaired the systemic immune response in young rats, hence predisposing them to parasitic infection in the gut(Menard et al. 2014a). More recently, we reported that perinatal exposure to BPA (50 µg/kg BW/d) induced intestinal and systemic immune imbalances in male offspring mice in adulthood through a decrease in Th1/Th17-cell frequencies in the lamina propria concomitant with an increase in the splenic Th1/Th17 immune response(Malaise et al. 2017). In comparison, the same BPA perinatal exposure led to a defect in dendritic cell (DC) maturation in the lamina propria and spleen associated with a decrease in activated and regulatory T cells in the lamina propria of female offspring mice. Moreover, a sharp increase in interferon-γ and interleukin-17 production by intestinal immune cells and a T helper 17 profile in the spleen were observed(Malaise et al. 2018). Dietary BPA intake causes a reduction in species diversity in the gut microbiome. Specifically, dietary BPA intake favours the growth of *Proteobacteria* and *Helicobacteraceae* and reduces the population of *Clostridia* in the gut microbiome(Lai et al. 2016). Moreover, dietary BPA uptake increases colonic permeability and disrupts the colonic chemical barrier(Feng et al. 2019).

However, the effects of oral BPA, which is the main route of BPA exposure, remain unclear, particularly at levels equivalent to human exposure. In the current study, we aimed to evaluate the effects of direct oral exposure of adult mice to low BPA doses relevant to human exposure in a murine model of food allergy to wheat. In this allergic model, we also investigated the effect of dietary exposure to BPA on the immune system, gut epithelial barrier function and gut microbiota.

2. Materials and methods

Animal model. Three-week-old female BALB/cJRj mice (Janvier Labs, Le Genest-Saint-Isle, France) were housed in a ventilated cage system and in specific pathogen-free conditions and used to obtain pups. After birth in our animal facility, the mice used in this study were exposed orally to different doses of bisphenol A diluted in water (Sigma–Aldrich, Saint Quentin Fallavier) at 0, 0.4, 4 and 40 µg/kg body weight/day by gavage solubilized in drinking water in nonfasted mice from Day 14 after birth to Day 49 corresponding to one day after the first allergic oral challenge. The mice were fed ab libitum with a gluten-free diet to avoid tolerance. On the 21st day, they were sensitized with wheat native gliadins (NGs) with alum hydroxide (1 mg/ml) by intraperitoneal injections separated by 10 days(Castan et al. 2018). One week after the last sensitization, the mice were orally administered 20 mg of NGs to induce the allergic reaction twice. The CTL mice were sensitized with alum only and challenged with PBS. CTL mice exposed only to BPA without being sensitized to food allergens were also included (supplemental figures). We measured body temperature by rectal probe 30 min after the last oral challenge.

ELISA. Blood was collected via cardiac puncture after the oral challenge and centrifuged at 1500 g for 15 min. The assay for the quantification of gliadin-specific Igs was performed in serum samples via indirect ELISA, as previously described(Gourbeyre et al. 2012). Specific Ig binding is expressed by the ratio of the mean fluorescence intensity measured with wheat native gliadins (IF) to the mean fluorescence intensity measured with carbonate buffer (IF0) as the background signal(Lupi et al. 2013). The results are shown as IF/IF0 corresponding to normalized values without background. Mouse mast cell protease-1 (mMCP-1) was measured in

serum using an ELISA kit (Ready Set Go, eBioscience) according to the manufacturer's protocol.

Intestinal permeability. Pieces of jejunum were extracted from the mice, washed with a Krebs solution and mounted in Ussing chambers (Physiological instruments, San Diego, CA, US). Paracellular permeability was assayed by measuring the flux of fluorescein–5.6 sulfonic acid (400 Da; 1 mg/mL, Invitrogen) for 150 min using a spectrofluorometer. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current with an automatic voltage clamp (Physiologic instrument, US). Tissue ion resistance (1/G), where G is the conductance, was calculated from the potential difference and short-circuit current according to Ohm's law using acquisition and analysis software (Physiologic Instrument, US).

Flow cytometry. The gut mesenteric lymph nodes (mLns) were removed and crushed to obtain a single-cell suspension, and the cells were suspended in PBS/5% FBS buffer. For the lamina propria, the intestine was flushed after removing the Peyer's patches. Then, the gut was opened longitudinally, cut into small pieces and digested with EDTA/HEPES/DPBS on a shaker at 37 °C for 15 min. After filtration through a 100-µm filter, Percoll was realized. Cells were stained with the following surface markers: CD3-FITC, CD11b-PerCP-Cy5.5, CD4-BV421, CD11c-BV510, CD25-BV510, CCR4-PE, CXCR3-APC, MHCII-FITC (BD Biosciences, Le Pont-de-Claix, France), Lineage-FITC, ST2-PE, DX5-PerCP5.5 and CD127-APC in the presence of CD16/32 mAbs (BD Biosciences). Cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and stained with anti-Foxp3-Alexa Fluor 488 and RoryT-PE (BD Biosciences). The cells were analysed on a FACS CANTO II (BD

Biosciences). Data were acquired using DIVA software (BD Biosciences) and analysed with FlowJo (TreeStar).

Cytokine quantification. Splenocytes were stimulated for 5 hours with 100 µg/mL gliadin with brefeldin A (Golgi plug, BD Biosciences, 1:1000). Cytokine concentrations in supernatants were quantified by Luminex technology (BioPlex 200 system, Bio-Rad Laboratories, Munich, Germany) using a Pro Mouse Group I Cytokine 23-plex kit (Bio-Rad Laboratories). Assays were performed according to the manufacturer's specifications.

Caecal analyses. The caecal content from mice was collected, frozen and centrifuged at 10,000 *g* for 10 min. The bacteria in the caecal contents were quantified by quantitative PCR (qPCR). Briefly, the caecal content was added to 180 µl of a solution of lysozymes (20 mg/ml), Triton (1.2%) and Tris–HCl/EDTA (1/0.5 M) and incubated at 37 °C for 30 min. Then, 300 mg of zirconium beads (diameter 0.1 mm, BioSpec Products, VWR International, Fontenay-sous-Bois, France) were added to 1 ml of InhibitEX solution from the QIAampbFast DNA Stool Mini Kit, and the suspension was disrupted for 3 min at 30 Hz in a mini-bead beater (MM301; Retsch GmbH & Co., Haan, Germany). Afterwards, the DNA was quantified by Nanovue (GE Healthcare, Aulnay sous Bois, France). Following extraction, qPCR was conducted using an iCycler iQ real-time PCR detection system instrument (Bio-Rad, Hercules, CA, US). The analysed mixture consisted of a total volume of 15 µl containing 5 µl of the extracted DNA, 2.5 µl of specific primers and 7.5 µl of the QuantiTect SYBR Green PCR Kit (Qiagen, Venlo, Netherlands).

Statistical analysis. Data were analysed using GraphPad Prism 6.0 (La Jolla, CA, US). Values are expressed as the mean ± SEM and were compared using one-way

ANOVA followed by Tukey's multiple comparison test. A *p value* below 0.05 was considered significant.

3. Results

Bisphenol A exposure increases food allergy symptoms in mice

To evaluate the effect of direct oral exposure of adult mice to a low dose of BPA on the development of allergies, we used a murine model of food allergy to wheat **(Figure 1A)**. To characterize the allergic symptoms, we measured the body temperature after the second oral challenge. As seen in **Figure 1A**, we analysed the allergic reaction after a second oral challenge occurring two days after the arrest of BPA exposure. Our results showed a significant decrease in body temperature after challenge in mice sensitized with native gliadin (NG). This temperature drop was also observed during the direct oral exposure to BPA at any dose without being significantly different between food allergy (FA) and FA exposed to BPA (0.7 ± 1.2 °C vs. -1.7 ± 0.3 °C in CTL and FA, respectively) **(Figure 1B)**. Nevertheless, the temperature drop in mice exposed to BPA at doses of 4 µg/kg and 40 µg/kg tended to be aggravated without reaching significance **(Figure 1B)**. Moreover, we observed a significant increase in the plasma level of mMCP-1 in allergic mice compared to CTL mice (333 ± 52 pg/ml vs. 516 ± 64 pg/ml in CTL and FA, respectively) **(Figure 1C)**. Interestingly, the plasma level of mMCP-1 was increased in mice exposed to BPA at 4 µg/kg (FA 4 BPA) and 40 µg/ml (FA 40 BPA) compared to allergic mice (FA) **(Figure 1C)**. When measuring the level of wheat-specific IgE in the blood of mice after the second challenge, as expected, we observed an increase in specific IgE in FA mice compared to CTL mice (1 ± 0.04 AU vs. 4 ± 0.51 AU) **(Figure 1D)**. Interestingly, mice exposed to BPA displayed a higher increase characterized by a

more elevated level of specific IgE than FA mice, regardless of the dose of exposure **(Figure 1D)**. As a control, the effect of BPA alone on nonallergic mice at the three doses was also realized and did not show allergic symptoms or modification of allergic markers, including MCP-1 and specific IgE plasma levels, compared to unexposed mice **(data not shown)**. Taken together, these results demonstrate that direct oral exposure, especially the TDI dose or higher of BPA, strengthens allergic markers, such as specific IgE and mMCP-1.

Bisphenol A exposure increases gut permeability and resistance in a dose-dependent manner

To assess whether exposure to BPA alters intestinal barrier functions induced by food allergy, we evaluated intestinal paracellular permeability and tissue resistance using Ussing chamber analyses after the second challenge **(Figure 2)**. Our results showed an increase in acid sulfonic FITC flux in allergic mice and allergic mice exposed to BPA compared to CTL mice **(Figure 2A)**. As a control for the BPA effect in naïve mice, we observed an increase in acid sulfonic FITC flux in association with exposure to BPA only at the highest dose of 40 µg/kg **(Supplementary Figure 1A).** The area under the curve demonstrates that mice exposed to 4 µg/kg and 40 µg/kg FA displayed an increase in acid sulfonic FITC flux compared to FA mice (1095 ± 272 AU vs. 1518 ± 318 AU vs. 461 ± 36 AU in FA 4 BPA, FA 40 BPA and FA, respectively), suggesting an aggravation of gut leakage induced by food allergy **(Figure 2B)**. Conversely, the measurement of tissue electrical resistance showed a decrease in FA mice compared to CTL mice (87 ± 29 $\Omega.cm^2$ vs. 116 ± 30 $\Omega.cm^2$ in FA and CTL mice, respectively) **(Figure 2C)**. Moreover, this decrease was even more pronounced in FA 4 BPA mice and FA 40 BPA mice than in FA mice, in accordance with the results observed for gut permeability **(Figure 2C)**. Gut permeability in mice

exposed to BPA alone was increased, which was associated with a decrease in gut resistance in mice exposed to BPA alone at a dose of 40 µg/kg without food allergy **(Supplementary Figure 1B)**. These results suggest that an increase in intestinal epithelial layer permeability at doses of 4 and 40 µg/kg BPA is associated with a decrease in electrical resistance.

BPA exposure modulates the humorall response in a dose-dependent manner

To compare the development of the humorall response among the different groups, the concentrations of gliadin-specific IgG1, IgA and IgG2A were determined after the second challenge **(Figure 3)**. We observed a significantly higher level of specific IgG1 in the four groups of FA mice than in the CTL mice (10 ± 0.11 AU vs. 1 ± 0.08 AU in FA and CTL mice, respectively) **(Figure 3A)**. However, no differences were found between the FA group and the FA group exposed to BPA **(Figure 3A)**. For the level of specific IgA, we observed a significant increase for all groups of FA mice compared to CTL mice *(*4.3 ± 0.94 AU vs. 1.1 ± 0.07 AU in FA and CTL mice, respectively) **(Figure 3B)**, with the highest level in the FA 4 BPA and FA 40 BPA groups. The IgA levels of the FA 4 BPA and FA 40 BPA groups were increased compared to those of the FA groups. However, the level of specific IgA was not different between FA mice and FA mice exposed to BPA at doses of 0.4 µg/kg **(Figure 3B)**. In contrast, FA mice displayed a higher level of specific IgG2A than CTL mice (7.9 vs. 0.9, $p<0.005$ for FA and CTL, respectively) **(Figure 3C).** In line with our previous results, FA 4 BPA and FA 40 BPA showed decreased levels of specific IgG2A compared to FA mice or FA 0.4 mice **(Figure 3C)**. The level of specific IgG2A was similar between FA mice and FA 0.4 mice **(Figure 3C)**. As expected, mice exposed to BPA alone did not display changes in the levels of specific IgG1, IgA and IgG2a, regardless of the dose used **(Supplementary Figure 1C-E)**. Altogether, our

results suggest that exposure to BPA, especially at a dose of 4 µg/kg and higher, induces a modulation of the humorall response towards a Th2 response highlighted by an increasing rate of IgG1 and IgA and a dampened Th1 response via a decreased rate of IgG2a.

BPA exposure alters the T-cell response towards sensitization

To characterize the immune response activated during sensitization and oral exposure to BPA, we analysed cell infiltration in the mLNs **(Figure 4)**. Therefore, we measured T cells Th1, Th2, Th17 and Treg subset frequencies based on surface makers or transcription factor expression using the gating strategy defined in **Figure 1A**(Bonecchi et al. 1998; Hori et al. 2003; Yang et al. 2008). According to our previous results, we observed a significant increase in the frequency of $CD3^+CD4^+CCR4^+$ Th2 cells in mLNs in FA mice compared to CTL mice **(Figure 4B)**. Moreover, this increase in Th2 cells was more pronounced in FA 4 BPA and higher mice than in FA mice. In contrast, the FA 0.4 BPA group showed levels of Th2 cells similar to those of the FA group **(Figure 4B).** In naïve mice, BPA exposure did not alter the frequency of Th2 cells **(Supplementary Figure 2A).** In contrast, after challenge, we observed a similar frequency of $CD3^+CD4^+CXCR3^+$ Th1 cells in mLNs in the CTL and all FA groups **(Figure 4C)**. Because Tregs are known to regulate Th1 and Th2 responses, we measured Tregs as defined by high CD4 and CD25 expression and Foxp3 expression in the mLNs of the different groups of mice **(Figure 4D).** A significant decrease was observed in the Treg frequency in FA mice compared to CTL mice **(Figure 4D).** A similar decrease was observed in FA mice exposed to BPA compared to CTL mice. Finally, no difference was found when comparing the FA group with the FA exposed to BPA group. Of note, BPA exposure decreased the Treg frequency in naïve mice at doses of 4 and 40 µg/kg but not at 0.4

µg/kg **(Supplementary Figure 2B).** As a more recent T cells subset implicated in inflammatory responses, such as allergic responses, we measured the frequency of the Th17 subset characterized by the expression of the nuclear expression of RAR-related orphan receptor gamma **(Figure 4E)**. As expected, FA mice displayed a higher frequency of Th17 cells than CTL mice. Interestingly, BPA exposure at doses of 4 and 40 µg/kg increased Th17 cells compared to the FA and FA 0.4 groups **(Figure 4E)**. In naïve mice, BPA exposure altered the frequency of Th17 cells only at the higher dose corresponding to 10 times the TDI **(Supplementary Figure 2C).** These results indicate that exposure to BPA might be directly responsible for the T-cell imbalance observed in mice and its consequences in the allergic response. The T-cell response is initiated by contact with antigen-presenting cells, especially DCs, which are regulated by plasmacytoid DCs (pDCs) and are largely influenced by the presence of innate cells, such as recently defined innate lymphoid cells (ILCs), which are the counterparts of T cells that contribute to immune responses. ILCs are particularly abundant at mucosal barriers, where they are exposed to allergens. Therefore, we measured the frequency of DCs and pDCs in mLNs and ILC1 s and ILC2s in the lamina propria of mice from each group **(Figure 5)** as defined by the gating strategy presented in **Figure 5A**. We observed a significant increase in total CD11c+MHCII+ DCs in all FA mice compared to CTL mice **(Figure 5B).** Similar results were obtained in naïve mice exposed to BPA at doses of 4 µg/kg and 40 µg/kg but not at 0.4 µg/kg **(Supplementary Figure 3D)**. Our results suggest that BPA exposure can modulate the immune response towards the inflammatory response by increasing antigen-presenting cells at doses equal to and superior to the TDI of 4 µg/kg and in turn may aggravate allergic reactions. In contrast, we did not find any modulation of plasmacytoid dendritic cells (pDC) thought to be able to

promote both T-cell activation and tolerance(Villadangos and Young 2008) in CTL mice compared to FA and FA exposed to BPA mice **(Figure 5C)**. Accumulating evidence suggests that ILCs together with DCs play substantial roles in protection against infection and the pathogenesis of inflammatory diseases, such as allergic and autoimmune diseases(Morita et al. 2016). Therefore, we investigated the effect of BPA exposure on innate lymphoid cells (ILCs) **(Figure 5D and E)**. We especially focused on the ILC2 and ILC1 subsets, which are critical in food allergy physiopathology. As expected, FA mice displayed an increase in gut ILC2s compared to CTL mice **(Figure 5D)**. Surprisingly, BPA-exposed mice at doses of 4 and 40 µg/kg also displayed an increase in their gut ILC2s compared to FA and FA 0.4 mice, which was already significantly different from CTL mice **(Figure 5D)**. In naïve mice, BPA exposure did not alter the frequency of ILC2s **(Supplementary Figure 3E)**. In contrast, food allergic mice did not display an increase in gut ILC1 compared to CTL FA-exposed BPA mice **(Figure 5E)**. Similarly, in naïve mice, BPA exposure did not alter the frequency of ILC1 cells **(Supplementary Figure 3F).** These results show that BPA exposure at a dose equal to or superior to the TDI is able to upregulate not only T helper cells but also DCs and ILCs in allergic diseases according to their subtype. To evaluate whether BPA exposure could modulate cytokine production by splenocytes, we measured the levels of the Th2 cytokines IL-13, IL-4 and IL-5 **(Figure 6A)** and the Th1 cytokines IFN-γ, IL-12 and IL-10 **(Figure 6B)** in the supernatant of splenocytes stimulated with allergens (wheat gliadins). According to our results on T-cell subset frequency modulation by BPA, we observed that Th2 cytokines, including IL-13, IL-4 and IL-5, were increased in FA mice compared to CTL mice (3.4 ± 0.8 pg/ml vs. 1.5 ± 0.2 pg/ml for IL-13, 47.9 ± 31.9 pg/ml vs. 1.13 ± 1.25 pg/ml for IL-4 and 4.9 ± 3.8 pg/ml vs. 2.0 ± 2.1 pg/ml for IL-5 in FA and CTL mice,

respectively). Moreover, the measured Th2 cytokines, including IL-13, IL-4 and IL-5, were significantly increased in FA 4 BPA compared to FA mice (7.5 ± 1.1 pg/ml vs. 3.4 ± 0.8 pg/ml for IL-13, 136.6 ± 18.2 pg/ml vs. 47.9 ± 31.9 pg/ml for IL-4 and 23.2 ± 13.8 pg/ml vs. 4.9 ± 3.8 pg/ml for IL-5 in FA 4 BPA and FA mice, respectively) **(Figure 6A)**. In contrast, Th2 cytokine levels were similar between FA mice and FA 0.4 and 40 BPA mice as well as between the three FA BPA groups. These results indicate that BPA exposure tends to aggravate Th2 cytokine production induced by food allergy, especially at a dose of 4 µg/kg. In contrast, the Th1 cytokines IFN-γ and IL-12 as well as IL-10 were increased in FA mice compared to CTL mice (3.4 ± 0.8 pg/ml vs. 1.5 ± 0.3 pg/ml for IFN-γ, 47.9 ± 31.9 pg/ml vs. 1.1 ± 1.2 pg/ml for IL-12p70 and 8.1 ± 0.9 pg/ml vs. 2.1 ± 2.1 pg/ml for IL-10 in FA and CTL mice, respectively) **(Figure 6B).** However, an IFN-γ increase was not observed in FA 4 BPA and FA 40 BPA mice **(Figure 6B).** IFN-γ levels were decreased compared to those in FA mice and reached a level similar to that observed in CTL mice. IL-12 levels remained unchanged between FA mice and FA BPA mice, regardless of the dose of BPA used **(Figure 6B).** Finally, IL-10 levels were increased in a similar manner in FA mice and in FA mice exposed to BPA compared to the CTL mice. Our results confirm the exacerbation of allergic inflammation by BPA exposure characterized by an increase in Th2 cytokine production, especially at doses relevant to human exposure (4 µg/kg bw/d) corresponding to the TDI. Moreover, the TDI or a higher dose of BPA dampened IFN-γ production, suggesting modulation of the Th1 response or an impact on the cellular source of IFN-γ.

BPA exposure alters gut microbiota diversity

To characterize the impact of oral exposure to BPA on gut microbiota known to be affected by FA, we sequenced 16S DNA from the caecal content in FA mice

exposed to BPA **(Figure 7)**. Alpha diversity was investigated to analyse the complexity of the species diversity(Schloss 2009) through several indices, including observed species and the Shannon index. The flattening of the rarefaction curve based on the values of observed species indicated that our data volume covered all species of the community in the gut samples in all groups **(Figure 7A)**. Moreover, we observed a decrease in gut bacterial operational taxonomic units (OTUs) in the FA and FA exposed to BPA mice, suggesting a decrease in species number (298 ± 20 OTUs vs. 360 ± 56 in FA and CTL mice, respectively) **(Figure 7B)**. Interestingly, the observed species number in the FA 4 BPA group was decreased compared to that in the FA group (225 ± 20 OTUs vs. 398 ± 20 OTUs in FA and CTL mice, respectively) **(Figure 7B)**. However, BPA exposure alone already affected OTU number in naïve mice characterized by a decrease in OTU number observed for mice exposed to BPA at 4 and 40 µg/kg but not for 0.4 µg/kg **(Supplementary Figure 3B).** To reflect how many different types of species were present in our group of mice, we measured the Shannon index, reflecting both the diversity and the richness of the community. Significant decreases in the Shannon index from both the FA and the FA BPA groups were detected compared to the CTL group (5.2 ± 0.4 OTUs vs. 6.5 ± 0.6 OTUs in FA and CTL mice, respectively) **(Figure 7C).** This effect was even more pronounced in the FA 4 BPA group than in the FA and FA 0.4 BPA groups. According to the observed OTUs, the Shannon index was also affected by BPA in naïve mice, with a decrease in the BPA 4 and BPA 40 groups but not in the BPA 0.4 group compared to CTL mice **(Supplementary Figure 3C).** A comparative analysis of the microbial communities in the different groups was conducted at the level of six main phyla: *Bacteroidetes, Actinobacteria, Firmicutes, Proteobacteria, Verrucomicrobia* and *Fusobacteria*. These phyla were chosen because they are known to be modulated in

allergies(Berin 2019; Bunyavanich and Berin 2019; Fieten et al. 2018) and by BPA(Feng et al. 2020; Lai et al. 2016; Reddivari et al. 2017). Distribution histograms of the taxonomic composition of the six main phyla from the respective groups were constructed **(Figure 7D).** In the FA groups, the gut microbial communities were significantly enriched in the phylum *Firmicutes* compared to the CTL group (56 ± 10 vs. 70 ± 6.2% in CTL and FA mice, respectively) and deprived of *Fusobacteria* (14.2 ± 5 vs. 8.2 ± 3.4% in CTL and FA mice, respectively). In the faeces of FA BPA-exposed mice, a noticeable decrease in the *Bacteroidetes* relative abundance was observed at doses of 4 and 40 µg/kg compared to the CTL group (24.2 ± 8.3 vs. 10.5 ± 6.4 vs. 12.7 ± 6.9 in CTL, FA 4 BPA and FA 40 BPA mice, respectively) **(Figure 7D)**. However*,* the relative abundance of *Bacteroidetes* was similar in FA and CTL mice (24.2 ± 8.3 vs. 15.2 ± 6.6 in CTL and FA mice, respectively, p=0.069). In contrast, *Proteobacteria* was increased in FA 4 BPA and FA 40 BPA mice compared to the CTL group (6 ± 3.6 and 5.2 ± 3.1 vs. 0.9 ± 0.5 in FA 4 BPA, FA 40 BPA and CTL mice, respectively). Furthermore*,* the relative abundance of *Proteobacteria* was found to be similar in the CTL, FA and FA 0.4 BPA mice **(Figure 7D).** Finally, the relative abundance of *Fusobacteria* was decreased in all FA groups compared to the CTL group (14 ± 2.5 vs. 8.2 ± 3.4 in the CTL and FA groups, respectively) **(Figure 7D).** BPA exposure also affected the bacterial community in naïve mice **(Supplementary Figure 3D).** Although no differences were found in BPA 0.4 mice compared to CTL mice, exposure to higher doses of BPA, especially 40 µg/kg, induced strong changes in the bacterial community. Among them, we observed an increase in *Firmicutes, Proteobacteria* and *Verrucomicrobia* associated with a decrease in *Actinobacteri*a and *Fusobacteria* **(Supplementary Figure 3D).** Collectively, our data suggested that either the FA or FA BPA protocol could cause a

reduction in the species diversity of the microbial community in the animal gut with an increase in the effect induced by BPA exposure, especially a dose corresponding to the TDI or higher.

4. Discussion

Our study demonstrates that exposure to doses of BPA at those used to establish the TDI has an adverse effect on allergic markers, gut physiology and microbiota. These effects are clearly dependent on the dose used and the parameters measured. We observed an aggravation of allergic markers at the TDI and a higher dose characterized by specific IgE production and MCP-1 plasma levels. However, a tendency towards temperature drop after oral challenge was observed, although it did not reach significance. This might be linked to the large variability in temperature drop between animals. We observed modulation of antibody production, including specific IgA and IgG2a, together with an increase in gut permeability and resistance in food-allergic mice exposed to a dose of BPA corresponding to the TDI and higher. Moreover, exposure to BPA induced a stronger Th2 response and associated cytokine production. Finally, BPA exposure and food allergy induce a dysbiosis by themselves that tends to be aggravated in food-allergic mice exposed to BPA. The study by Menard *et al.* (FASEB 2014) was considered the key study, as it measured the dose-related effects of BPA starting from 0.5 µg/kg BW/day. Here, we used three doses of BPA: one at a TDI of 4 µg/kg, a second 10 times higher and a third 10 times lower. In our model of food allergy to wheat gluten, we observed an increase in specific IgE that was more important in food-allergic mice exposed to BPA than in allergic mice. Similarly, a significant increase in OVA-IgE titers was also observed in mice exposed to BPA in a respiratory allergy model(Bauer et al. 2012) (O'Brien et al. 2014; Petzold et al. 2014). Several experimental studies

have demonstrated some type of immune effects of BPA after developmental exposure. However, in most studies, the doses used and those that caused immunological effects were above those utilized to derive the current lowered no observable adverse effect level (NOAEL) BPA TDI of micrograms per kilogram of body weight per day (EFSA report, 2015). Exceptions to this “high” dose include the publications of Menard *et al*. and Bauer *et al*., indicating adverse effects on the immune system at lower doses. While evaluating *in vivo* gut epithelial barrier responses to systemic BPA exposure, we found that BPA significantly promoted the increase in gut permeability induced by food allergy associated with a decrease in electrical resistance. Of particular interest is the observation that BPA impacts intestinal permeability at levels of the TDI (i.e., at exposure levels usually considered safe for humans). There is now growing evidence showing endocrine-disrupting effects of BPA at doses greater than the reference limits(Rubin et al. 2001). The present dose–response study on intestinal permeability shows a maximal effect of BPA at the TDI regarding electrical resistance. It is, thus, questionable whether daily gut exposure to low doses of BPA may have implications for human health. The humoral response is a key element for allergic sensitization. Here, we show that the levels of specific IgA and IgG2a are affected by BPA exposure. In particular, the modulation induced by food sensitization is aggravated by BPA exposure at the TDI dose, which is in line with previous results outside the allergy context(Goto et al. 2007). IgG2a is a representative Th1-type antibody; hence, the decrease in IgG2a associated with an increase in IgE was thought to be linked with a Th2 response and cytokine pattern. In fact, our study indicates that splenocytes from TDI BPA-exposed mice exhibited an increase in Th2 cell frequency and Th2 cytokine pattern when they were stimulated with a specific antigen compared to allergic mice not exposed to

BPA. In contrast, BPA had the opposite effect on specific IgA levels, as we observed an increase in specific IgA levels in TDI BPA-exposed mice compared to allergic mice. The increase in IgA levels can be linked to an increase in the Th2 response(Yamamoto et al. 1996) and aggravation of allergies(Kim et al. 2017b), underlying the exacerbating effect of BPA on allergies. Various effects of BPA on immune responses have been reported. Most of the reported effects are strong, but they often contradict each other. For example, studies have reported that administering BPA results in an augmentation of Th1-type immune responses, especially in immunized mice(Alizadeh et al. 2006; Yoshino et al. 2003). In contrast, it was reported that increased Th2-type cytokine production in CD4 T cells from immunized mice was observed under i.p. administration of BPA(Lee et al. 2003). Speculation regarding the contradictions among these reports focuses on issues, such as the differences among routes (oral or i.p.), solvents and the conditions of BPA administration in the study system. A previous study showed that administration of IL-17 as well as the transfer of Th17 cells have the potential to abolish the therapeutic effects of oral tolerance to antigen, thereby suppressing the expansion of Treg cells (Kawakami et al., 2012). Our study revealed a significant increase in Th17 cells in a dose-dependent manner in FA mice exposed to BPA. ILCs have been shown to play important roles in inflammatory diseases and the maintenance of barrier homeostasis. Previous work has demonstrated that several cytokines influence allergic disease in different ways(Stier and Peebles 2017). Many of these cytokines, previously considered to be Th-cell derived, have recently been shown to be produced by ILCs. Our study demonstrates that ILCs, particularly ILC2s, are increased by BPA exposure at high doses. ILC2s are the subset of ILCs most extensively studied in the context of allergic inflammation due to their production of

proallergic mediators, including IL-4, IL-5, IL-9 and IL-13. Varieties of environmental stimuli are known to activate ILC2s. The activation of ILC2s in tissues profoundly changes their surface phenotype, consistent with several feed-forward loops that may promote the allergic response. However, immune cells were measured in mesenteric lymph node and measuring effect of BPA in the tissue especially in cells in lamina propria, in Peyer's patches and on intraepithelial lymphocytes would represent of strong argument to improve our knowledge on direct relationship between BPA exposure and immune system. However, as both belongs to the GALT (The gut-associated lymphoid tissue) they share some immunological properties and often display similar activation with different degrees. Current evidence indicates that the composition of the gut microbes plays an important role in maintaining animal health, and microbial community imbalances can predispose individuals to certain diseases(Horai et al. 2015; Sun and Chang 2014). To elucidate the influence of dietary exposure to chemical contaminants on the gut microbial ecology, this study examined whether BPA exerts selective pressure on the gut microbiota, the pattern of which could be used as a microbial signature in relation to allergic markers. As a recently classified "obesogen", BPA has been found to perturb lipid metabolism and pancreatic β-cell functions(Guan et al. 2016; Williams et al. 2014). However, its effect on the gut microbial profile in allergies is still unknown, as is its dose-dependent effect. Using 16S rRNA sequencing, we characterized the bacterial communities in the guts of mice exposed to BPA. Our results consistently demonstrated that BPA exposure could alter the number of bacterial species in the gut. Whereas FA is known to cause a decrease in the species diversity of gut microbes, we successfully showed that dietary BPA associated with FA aggravates this phenomenon. In the comparative 16S rRNA gene sequencing analysis, we further highlighted that BPA

intake resulted in exacerbation of alterations in the abundance of select microorganisms of the microbiota. One of the major observations was the increase in *Proteobacteria*. This phylum is usually in low abundance in the gut of healthy humans, and its proliferation reflects instability of the gut microbial community, suggesting an aggravation of disease conditions, as observed in association with a high-fat diet or steatosis(Feng et al. 2020; Lai et al. 2016). Moreover, we observed that the BPA mice showed a significantly increased percentage of *Verrucomicrobiota* in their faeces. As demonstrated by other studies, the level of *Verrucomicrobiota* in the intestines is associated with inflammatory bowel disease (IBD)(Carbonero et al. 2012). Although the exact mechanisms and pathology of IBD are still unknown, both the immunological response to the normal residential flora and the local inflammatory response may be contributing factors(Burich et al. 2001). Furthermore, we showed that BPA caused decreases in the phylum *Firmicutes*, of which most of the 16S rRNA belonged to the class *Clostridia*. Patients suffering from T2D reportedly harbour lower amounts of *Clostridia*(Snedeker and Hay 2012), and the proportion of *Firmicutes* and *Clostridia* in these diabetic patients is significantly lower than that in healthy individuals(Larsen et al. 2010). This observation again relates to the possible risk of metabolic disturbance associated with BPA uptake via alteration of the gut microbial ecology. In conclusion, our findings provide evidence of the negative effects of BPA in a wheat-induced food allergy mouse model. BPA can exacerbate allergic markers, increase serum levels of specific IgE and compromise Th2 responses. Moreover, the effect of BPA is linked to the dose used and affects gut physiology and microorganisms. Whereas these findings will need further verification in future studies and application to other bisphenols currently used, such as bisphenol F, our data highlight concerns about the potential health effects of BPA exposure. Furthermore,

our results serve as a warning regarding the adverse effects of BPA and the tolerable dose. The effects of BPA on the developmental immune system are consistent with the general concept and weight of evidence demonstrating the relatively high sensitivity of the developing immune system to perturbation by various toxicants, the consequences of which are often not discerned until later in life. Here, mice were exposed during the postnatal period (until 2 weeks of life), and it was shown that BPA evoked a decrease in paracellular permeability in adulthood, whereas the proinflammatory response of the colonic mucosa was strengthened(Braniste et al. 2010). Moreover, a study demonstrated that following gestational and lactational exposure to a low dose of BPA, Th17 cells in childhood and adulthood were dysregulated together with their transcription factors and regulatory cytokines(Luo et al. 2016). When considered in their totality, these studies suggest that current regulatory hazard and risk assessment strategies may not give sufficient attention to the possible developmental immunotoxicity of chemicals. Accumulating evidence points to the need to reconsider regulatory requirements for developmental immunotoxicity testing. With regard to BPA, growing evidence in the context of related animal studies clearly warrants reconsideration of the 2015 EFSA TDI.

Ethics approval and consent to participate

Experiments were approved by the ethics committee of Pays de la Loire (CEEA) under authorization number 13452.

Consent for publication

The authors have given their consent for publication.

Availability of data and materials

All data are available.

Competing interests

The authors have declared that no conflicts of interest exist.

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Authors' contributions

BMA performed the experiments, analysed the data and wrote the manuscript. MDC, ED and MK provided support for the mouse experiments and analysed the results. ED analysed microbiota sequencing. GB and LG designed and elaborated upon the study and supervised manuscript writing. MB and CB helped with writing the manuscript.

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Figure legends

Figure 1: ***Exposure to bisphenol A amplifies allergic markers***. (A) During direct exposure to low BPA doses (0.4, 4 or 40 µg/kg of bw/day), mice were sensitized by intraperitoneal injection of native gliadins (NGs) and then challenged twice with NGs by gavage. (B) Measures of body temperature 45 min after the second oral challenge. (C) Concentration of MCP-1 (pg/ml) in the plasma of mice after the second oral challenge. (D) Measures of wheat-specific IgE in the serum of the five groups of mice after challenge. CTL, black circle; FA, white triangle; FA 0.4 BPA, light grey triangle; FA 4 BPA, grey triangle and FA 40 BPA, dark grey triangle. Data represent the mean ± SEM (n = 6-8 animals per group); *p < 0.05, **p < 0.01 and ***p < 0.005.

Figure 2: ***Bisphenol A exposure increases gut permeability and resistance in allergic mice.*** Jejunum segments were extracted 1 hour after the second challenge, and (A) the intestinal paracellular permeability to FITC, (B) the area under the curve of the intestinal paracellular permeability and (C) the electrical resistance of the tissue ($\Omega.cm^2$) were measured using a Ussing chamber over a 2.5-h period. CTL, black circle; FA, white triangle; FA 0.4 BPA, light grey triangle; FA 4 BPA, grey triangle and FA 40 BPA, dark grey triangle. Data represent the mean ± SEM (n = 6-8 animals per group); *p < 0.05, **p < 0.01, ***p < 0.005 and ****p < 0.001.

Figure 3: ***BPA exposure modulates the humorall response in allergic mice.*** Measurements of wheat-specific (A) IgG1, (B) IgA and (C) IgG2A in serum from the five groups (CTL, black circle; FA, white triangle; FA 0.4 BPA, light grey triangle; FA 4 BPA, grey triangle and FA 40 BPA, dark grey triangle) of mice after the second

challenge. Data represent the mean ± SEM (n = 6-8 animals per group); *p < 0.05, **p < 0.01 and ***p < 0.005.

Figure 4: ***BPA exposure alters the T cells in a food allergy model.*** (A) Gating strategy to identify Th1, Th2, Treg and Th17 subsets. Frequency of (B) Th2, (C) Th1, (D) Treg and (E) Th17 cells in mLNs after the second challenge obtained by flow cytometry from the five groups of mice. CTL, black circle; FA, white triangle; FA 0.4 BPA, light grey triangle; FA 4 BPA, grey triangle and FA 40 BPA, dark grey triangle. Data represent the mean ± SEM (n = 6-8 animals per group) *p < 0.05, **p < 0.01 and ***p < 0.005.

Figure 5: ***BPA exposure alters the innate cell response in a food allergy model.*** (A) Gating strategy to identify DC, pDC, ILC2 and ILC1 cell subsets. Frequency of (B) DCs, (C) pDCs in mLNs, (D) ILC2s and (E) ILC1 cells in gut lamina propria after the second challenge obtained by flow cytometry from the five groups of mice. CTL, black circle; FA, white triangle; FA 0.4 BPA, light grey triangle; FA 4 BPA, grey triangle and FA 40 BPA, dark grey triangle. Data represent the mean ± SEM (n = 6-8 animals per group) *p < 0.05, **p < 0.01 and ***p < 0.005.

Figure 6: ***BPA exposure alters the cytokine response in a food allergy model***. (A) Th2 cytokine IL-13, IL-4 and IL-5 levels and (B) Th1 cytokine IFN, IL-12 and IL-10 levels in the supernatant of splenocytes stimulated with allergens from the five groups of mice. Data represent the mean ± SEM (n = 6-8 animals per group) *p < 0.05, **p < 0.01 and ***p < 0.005.

Figure 7***: Changes in the number and diversity of gut microbial species by BPA exposure in FA mice***. Rarefaction curves (A) based on the observed species value indices were used to show that the data volume covered all species in the gut microbial community. The changes in (B) observed species number and (C) Shannon

diversity demonstrate that FA and BPA uptake reduces the species diversity of the gut microbial community. Changes in gut microbial communities at different taxonomic levels by bar charts (D) showing the changes in the relative abundances of *Bacteroidetes, Actinobacteria, Firmicutes, Proteobacteria, Verrucomicrobia* and *Fusobacteria* (n = 6-8 animals per group).

Figure 1

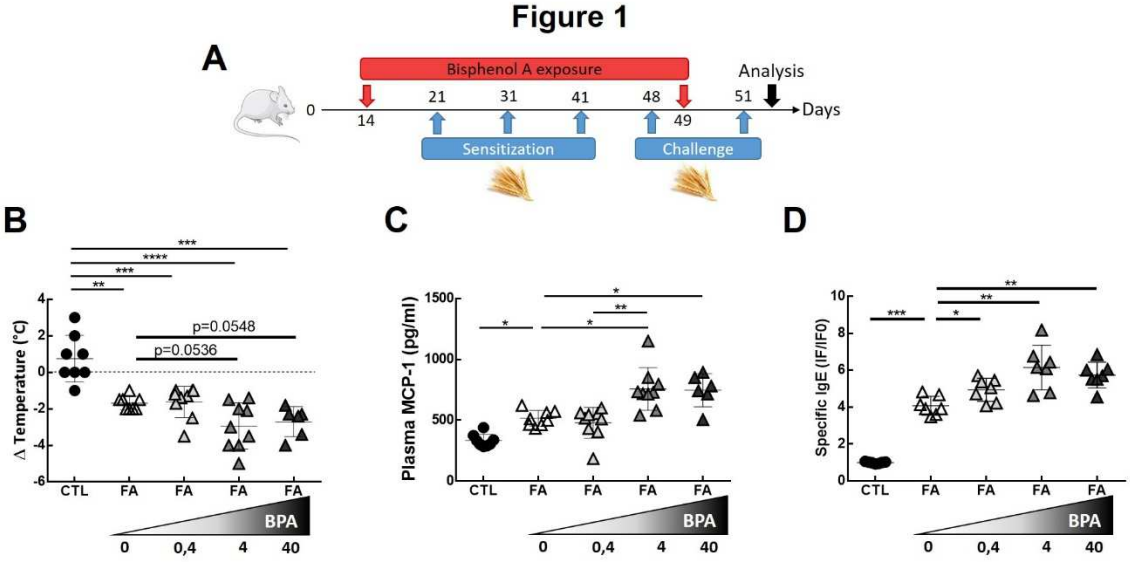


Figure 2

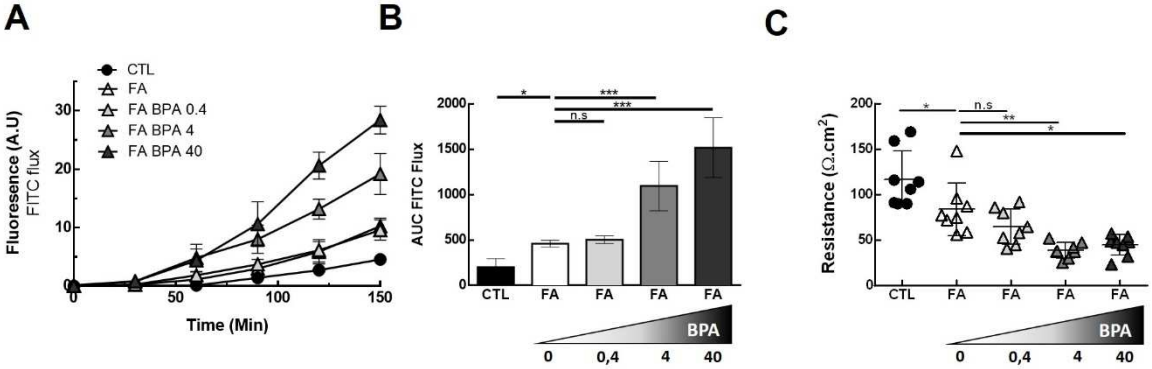


Figure 3

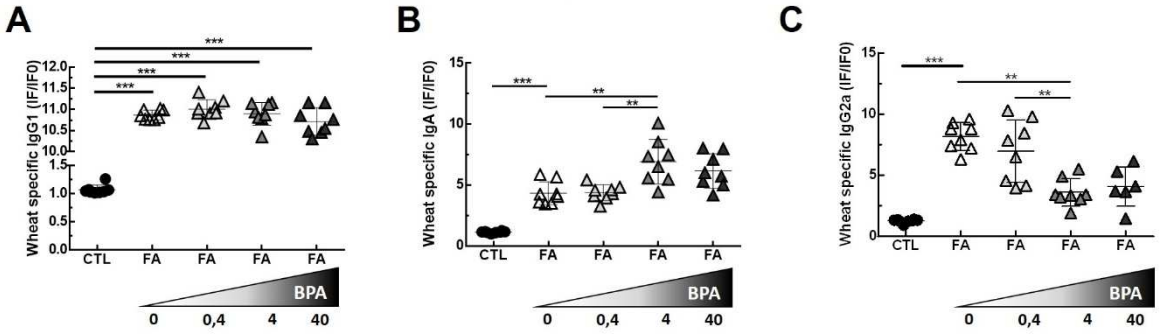


Figure 4

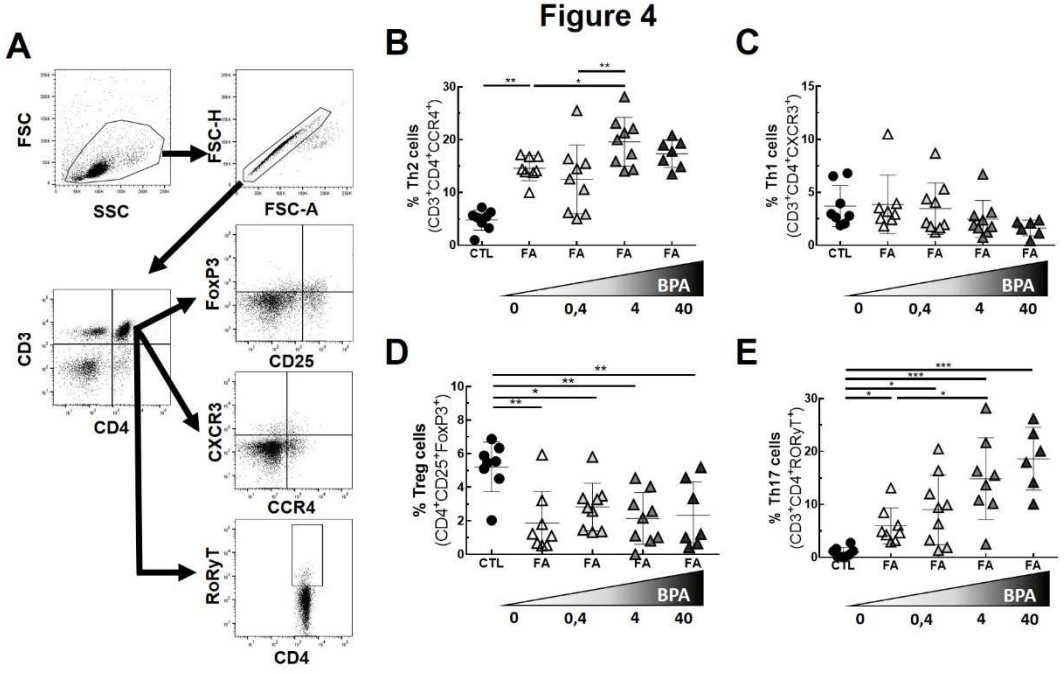


Figure 5

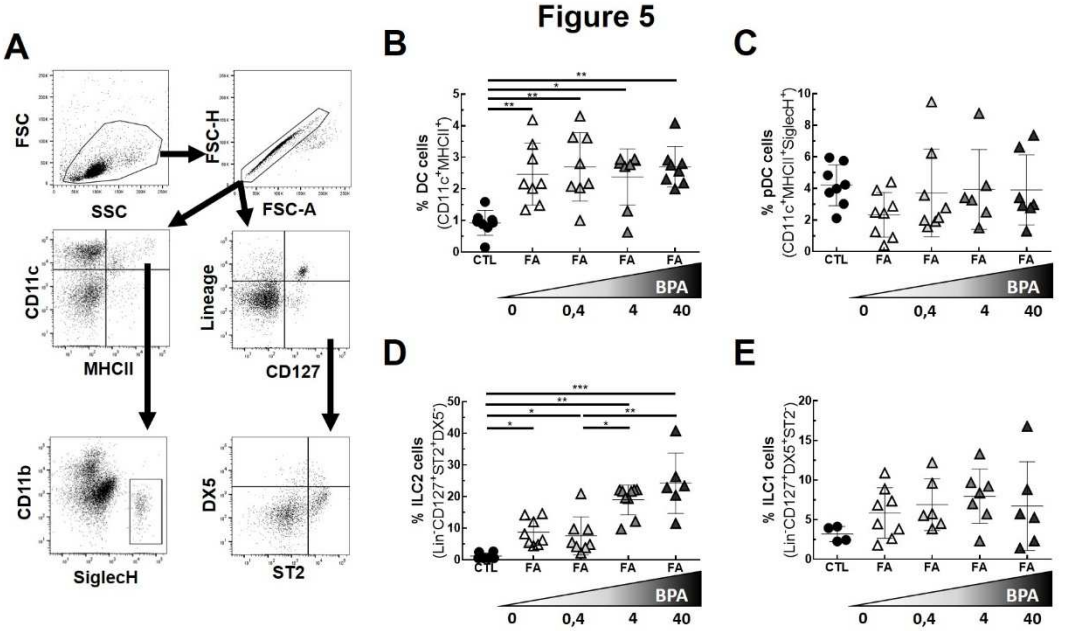


Figure 6

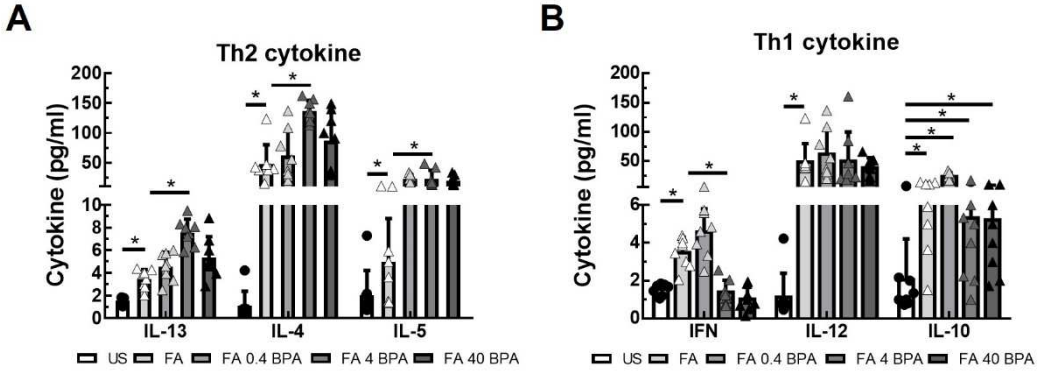
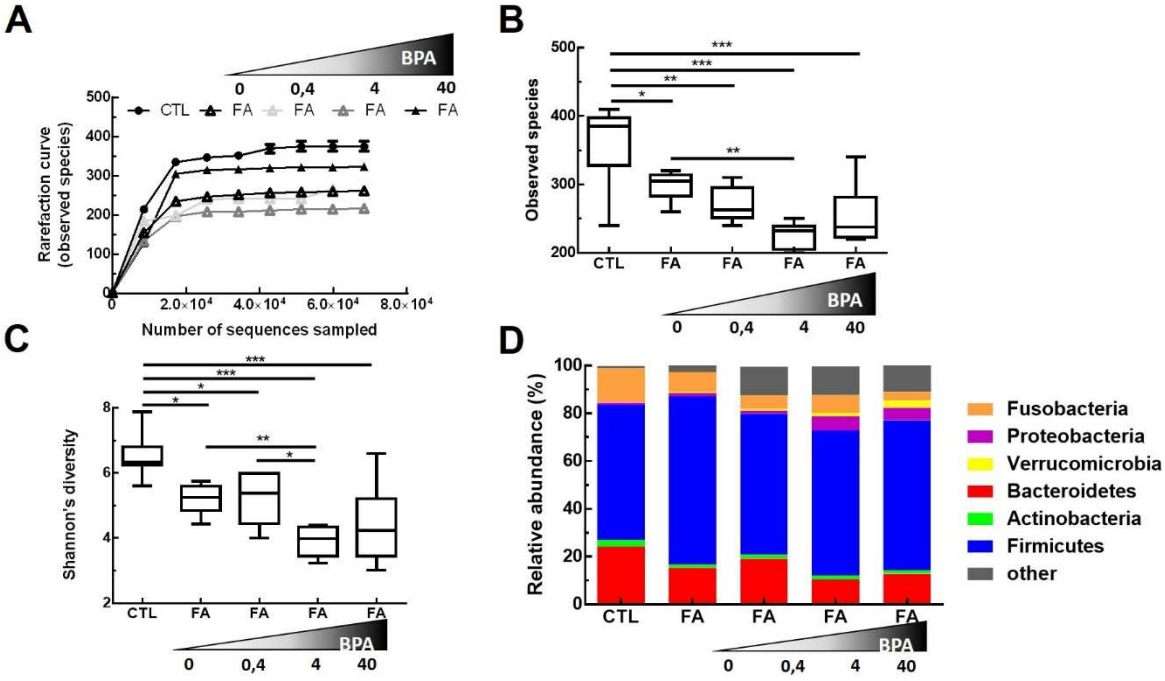


Figure 7


A
BPA
0 0,4 4 40
CTL FA FA FA FA
Rarefaction curve (observed species)
Number of sequences sampled
B
Observed species
CTL FA FA FA FA
BPA
0 0,4 4 40
C
Shannon's diversity
CTL FA FA FA FA
BPA
0 0,4 4 40
D
Relative abundance (%)
CTL FA FA FA FA
BPA
0 0,4 4 40
Fusobacteria
Proteobacteria
Verrucomicrobia
Bacteroidetes
Actinobacteria
Firmicutes
other