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ARTICLE

Immunoprogramming by Maternal Fibre and Polyphenol Intake Enhances Humoral Systemic and Mucosal Immunity in Adult Offspring

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Maternal diet is an element of the exposome that could shape the immune development in mothers' offspring. Although diets containing fibre and polyphenols are known for their direct immunomodulatory properties, their influence on the immune status of the adult offspring of mothers following such diets remains unclear. This preclinical study aimed to establish whether maternal consumption of a fibre- and polyphenol-enriched (FP) diet during a period including pregestation, gestation, and lactation modulates the immune response to a specific antigenic challenge in the offspring later in life. For this purpose, female Wistar rats were fed either a reference or FP diet across the above three periods. Their offspring, fed with a standard diet after weaning, were immunized with ovalbumin (OVA) at the age of seven weeks. Offspring from FP-fed mothers exhibited a markedly enhanced humoral immune response, characterized by elevated levels of anti-OVA antibodies in both systemic and mucosal compartments. No significant differences were observed in the delayed-type hypersensitivity (DTH) response. Moreover, FP offspring displayed a differential immunoglobulin (Ig) profiles in plasma and mesenteric lymph nodes. In parallel, an FP maternal diet changed the lymphoid composition in both the spleen and mesenteric lymph nodes of the offspring. In particular, there was a relative increased proportion of CD8+ cells in TCR $\alpha\beta$ + and NKT subsets. These findings provide evidence that a maternal diet enriched with fibre and polyphenols can durably program offspring immunity, enhancing their capacity to mount more efficient humoral systemic and mucosal responses in adulthood.

1. Introduction

The exposome includes the full range of environmental exposures that a person experiences from conception onward, all of which can influence their health (1). Most studies focus on long-term chemical exposure in adult life, while evidence shows that some conditions during preconception, gestation, and lactation can influence the health development of offspring once they have reached adulthood (2).

The exposome influences multiple physiological systems, including metabolic, cardiovascular, neurological, and immune systems, through diverse molecular pathways (3). Among them, epigenetic mechanisms, such as DNA methylation and histone modifications, play a central role in translating environmental signals into long-lasting biological effects (4,5). Although much attention has been

paid to its influence on the metabolic and endocrine systems (6,7), growing evidence highlights the immune system as a key target of exposome-driven modulation (8). Among the environmental factors included in the exposome, diet stands out as a key modifiable determinant of health (6,9).

Diets rich in fibre and polyphenols have been associated with beneficial effects on metabolic regulation, immune homeostasis, and inflammation control (10,11). Fibre, which is primarily composed of nondigestible carbohydrates, promotes gut health by modulating microbiota composition and increasing the production of short-chain fatty acids (SCFAs), which supports mucosal immunity, reduces systemic inflammation, and promotes regulatory T cell populations (12–14). Clinical studies have shown that a high fibre intake correlates with lower levels of systemic inflammatory markers and improved vaccine responses (15). Polyphenols, a diverse group of plant-derived compounds, including flavonoids, phenolic acids, and lignans, exhibit antioxidant, anti-inflammatory, and immunomodulatory properties (16), and some of them also can act as prebiotics by fostering beneficial microbiota and influencing the production of immunologically active microbial metabolites (17,18). Preclinical studies have demonstrated that certain polyphenol-rich diets can modulate dendritic cell activation, promote a regulatory immune environment, and reduce allergic sensitization (19,20). In

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addition clinical studies report improvements in the outcomes of allergic diseases, such as asthma and allergic rhinitis (19,20). These findings consolidate the important role of dietary fibre and polyphenols in modulating immune responses, inflammatory status, and the capacity to mount effective and regulated antigen-specific immune reactions.

Nowadays, the critical role of maternal nutrition during sensitive periods such as gestation and lactation in shaping both the mother's and the offspring's metabolic and immune health is clear (21). For instance, the maternal diet during gestation can influence foetal growth, adiposity, glucose metabolism, and microbiota development, while nutrition during lactation may affect the composition of breast milk, the transfer of bioactive molecules, and early microbial colonization of the infant gut, all of which contribute to immunological and metabolic outcomes (22–24). However, despite increasing interest, fewer studies have investigated the impact of nutrition during the preconception period, a crucial but underexplored window of developmental plasticity. Preliminary evidence suggests that dietary exposures during pregestation can influence oocyte quality, uterine immune readiness, and epigenetic programming, which may persist through gestation and impact the health of offspring later in life (25).

It has been shown that maternal intake of fibre, polyphenols, or both during gestation and lactation modifies the composition and activity of gene expression and the gut microbiota, and enhances the quality of breast milk (26–28). For example, preclinical studies have demonstrated that maternal fibre supplementation during pregnancy increases the abundance of SCFA-producing bacteria, promoting regulatory immune responses and protecting offspring from infant allergic disease (29). The maternal influence on the microbiota and breast milk composition extends to the functionality of the neonatal immune system (30,31). Similarly, polyphenol intake during pregnancy and lactation has been linked to enhanced mucosal immunity and the modulation of immune cell differentiation in offspring (32). In a clinical context, maternal adherence to a diet rich in fibre and polyphenols during pregnancy was associated with beneficial Ig and cytokine profiles in cord blood, suggesting a programming effect on the neonatal immune system (33). Together, these findings support the concept of maternal immunonutrition as a strategy for shaping immunoprogramming in offspring.

However, despite evidence of the benefits of a maternal diet in terms of early immune development, the extent to which a maternal diet enriched in fibre and polyphenols can influence specific immune challenges in offspring later in life remains unclear. In the present study, we hypothesize that maternal intake of fibre and polyphenols beginning before gestation and lasting until the end of the lactation period may exert long-term immunoprogramming effects on offspring that persist into adulthood. Therefore, the aim of this study was to ascertain, in a preclinical model, whether such a maternal dietary intervention influences the specific immune response of offspring to an antigenic challenge later in life at both systemic and mucosal immune system.

2. Experimental

2.1. Diets

A standard diet (REF diet) based on the AIN-93G formulation (34) (Envigo, Indianapolis, IN, USA) and a fibre and polyphenol-enriched diet (FP diet) were used (Table 1). The FP diet contained 8 % inulin (FIBRULINE™, derived from chicory roots, Cosucra, Warcoing, Belgium) and 1 % pectin (PE21006 from citrus fruits, Gojira Fine Chemicals, LLC, Mundelein, IL, USA) as additional fibre sources, and 0.5 % polyphenols including catechin (C1251), epicatechin (E1753), hesperidin (H5254), naringenin (N5893), and quercetin (Q4951) (all sourced from Millipore Sigma, Madison, WI, USA). The pelleted diets were produced vacuum-packed to prevent oxidation and contamination by Envigo, and stored at 4 °C until use. The types and doses of fibre and polyphenol in the FP diet were established in accordance with the content of a healthy dietary pattern in a gestating/lactating Spanish Mediterranean cohort (35), and the doses of these components were established based in previous preclinical approaches (36).

2.2. Animals: mothers and offspring

Six-week-old female and male Wistar rats were obtained from Janvier Labs (Saint-Berthevin, France) and housed in the experimental animal facility of the Diagonal Campus at the University of Barcelona (UB). Female rats (n = 6 females/group) were randomly assigned to the REF group, fed the REF diet, or to the FP group, fed the FP diet. The diets were provided before gestation (3 weeks), during mating with a male (4 days), during gestation (3 weeks) and during lactation (3 weeks). The pups were born naturally and on the same birth day, each litter was standardized to include 4 female and 4 male pups. Until day 21, the pups had unrestricted access to their mother's milk for nursing. On weaning day, 3–4 pups per dam were randomly selected from each litter (6 litter per diet), resulting in 18–24 offspring animals per group with a balanced sex distribution. All selected pups were kept on the REF diet until the end of the study. The required sample size was determined using the Appraising Project Office's program from the Universidad Miguel Hernández de Elche (Alicante, Spain). This calculation, based on intestinal IgA as variable, among others, assumed no dropout rate and a two-sided type I error of 0.05. Additionally, the sample size was adjusted in accordance with to the University Ethical Committee guidelines, to have animals from more than 5 different litters and adhering to the Three Rs principle for animal experimentation.

The animals were maintained under controlled environmental conditions (50–55 % humidity, 21 ± 2 °C, and a 12 h light–dark cycle), and they had *ad libitum* access to food and water. The offspring body weight was monitored throughout the study from their birth day to their last day.

All animal procedures were approved by the Ethics Committee for Animal Experimentation of the University of Barcelona (CEEA/UB, ref. 240/19) and the Generalitat de Catalunya (DAAM10933).

2.3. Immunization with ovalbumin



At seven weeks of age, 19 animals born from mothers fed the REF diet and 20 born from mothers fed the FP diet were distributed into two subgroups: 12 were immunized (named the REF OVA group and the FP OVA group, according to their mothers' diet) and 7-8 served as nonimmunized controls (named the REF group and the FP group, again depending on their mother's diet). Immunization was carried out with ovalbumin (OVA, grade V, Sigma-Aldrich, Madrid, Spain) emulsified in alum (Pierce, IL, USA) via the intraperitoneal (i.p.) route (500 μ L/rat containing 50 μ g of OVA and 20 mg of alum). Nonimmunized controls received the same volume of phosphate-buffered saline (PBS) via the i.p. route. After this single injection, immunization was followed over a four-week period, during which all animals (immunized and controls) were monitored, and blood samples were collected, from a saphenous vein at weeks 0, 2, 3, and 4 post-immunization as previously described (37).

2.4. Delayed-type hypersensitivity (DTH) response

After 4 weeks of immunization, 24 h before euthanasia, the thickness of both ears of each animal was measured using a 7309 pocket thickness gauge (Mitutoyo, Hampshire, UK) to establish baseline values. The animals were immediately anesthetized with isoflurane (Abbott Laboratories, Berkshire, UK), and 50 μ L of 1 mg/mL OVA solution in sterile PBS were injected into the right ear. The same volume of PBS was then injected into the left ear. After 24 h, ear thickness was measured again. In addition, biopsies from the ears of anesthetized rats were excised using a fixed-diameter punch, enabling the collection of ear samples with identical circular areas, which were immediately weighed. The results are expressed in terms of both the thickness and weight of the OVA and PBS ears of rats.

2.5. Sample collection

After 4 weeks of immunization, animals were anesthetized using ketamine (90 mg/kg) and xylazine (10 mg/kg) (Bayer A.G., Leverkusen, Germany). Measures for calculating the body mass index (BMI) ($\text{weight}/\text{length}^2$ (g/cm^2)) and the Lee index ($\text{weight}^{0.33}/\text{length} \times 1000$ ($\text{g}^{0.33}/\text{cm}$)) were carried out.

Cardiac exsanguination was conducted to collect blood samples. EDTA-treated blood samples were centrifuged to obtain plasma. Mesenteric lymph nodes (MLNs), thymus, spleen, liver, brain, heart, kidney, submaxillary salivary gland and small and large intestines were collected. Some samples were weighed and, in some cases, immediately processed or stored at -20 °C or -80 °C for future analysis.

A central section of the small intestine (SI) was longitudinally opened, weighed and incubated with PBS while shaking at 37 °C for 10 min to collect the gut wash (GW) as in previous studies (38).

Spleen, some MLNs, and thymus were processed as previously described to obtain lymphocytes (39). Briefly, tissues were ground using a 40 μ m mesh cell strainer (Thermo Fisher Scientific, Barcelona, Spain) and Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Madrid, Spain), enriched with 10% foetal bovine serum (FBS, Sigma-Aldrich), 100 IU/mL of streptomycin-penicillin (Sigma-Aldrich), 2 mM of L-glutamine (Sigma-Aldrich), and 0.05 mM

of 2- β -mercaptoethanol (Merck Millipore, Darmstadt, Germany). For lymphocyte isolation from spleen, erythrocytes were removed via an additional osmotic lysis step (40). Cell count and viability were subsequently assessed using the Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA).

Other MLNs were stored at -20 °C and later homogenized for Ig quantification as previously described (41).

2.6. Lymphocytic composition in spleen, MLNs, and thymus

Spleen, MLNs and thymus lymphocyte subsets were characterized by flow cytometry analysis using mouse anti-rat monoclonal antibodies (mAbs) conjugated to fluorochromes, as previously described (40). The following mAbs were used: CD4 (OX-35), CD8 α (OX-8), TCR $\alpha\beta$ (R73), and CD161a (10/78) from eBioscience (Frankfurt, Germany); CD8 β (341), TCR $\gamma\delta$ (V65), CD25 (OX-39), CD44 (OX-49), and CD103 (OX-62) from BD Biosciences (San Diego, CA, USA); and CD45RA (OX-33) from Caltag (Burlingame, CA, USA). These mAbs were conjugated to one of the following fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), or brilliant violet (BV) 421, BV 605, or BV786.

A negative control staining using isotype-matched control mAbs was included in all samples. Additionally, a pool of cells was stained with each single fluorochrome-labelled mAb as positive control. Data were acquired using an Aurora Cytek spectral flow cytometer (Cytek, Fremont, CA, USA) of the Flow Cytometry Unit (FCU) in the Cytometry Service of the Scientific and Technological Centres of the University of Barcelona (CCIT-UB, Barcelona, Spain) and analysed using FlowJo v.10 software (Tree Star, Inc., Ashland, OR, USA). Results are expressed as percentages of positive cells within the total lymphocyte population, gated based on forward- and side-scatter characteristics (FSC and SSC), or within a specific subpopulation.

2.7. Quantification of plasma and mucosal Ig concentration

For plasma and homogenized MLNs, a ProcartaPlex™ Multiplex immunoassay was used to quantify the concentrations of IgA, IgM, and IgG1, IgG2a, IgG2b, and IgG2c. The assay was carried out following the manufacturer's guidelines and as previously described (41). Results were obtained using a MAGPIX® analyser (Luminex Corporation, Austin, TX, USA) at the CCIT-UB. The sensitivity of the assay was as follows: 0.15 ng/mL for IgM; 1.16 ng/mL for IgG1; 2.08 ng/mL for IgG2a; 2.68 ng/mL for IgG2b; 4.21 ng/mL for IgG2c and 0.46 ng/mL for IgA.

2.8. Quantification of plasma and mucosal anti-OVA antibodies

To determine the levels of specific anti-OVA antibodies (Abs) in plasma, GW, and homogenized MLNs, an indirect ELISA was performed as previously described (42). In addition, the concentrations of specific anti-OVA IgG1, IgG2a, IgG2b, and IgG2c Abs were also determined (43).

Briefly, plates were coated with OVA (10 μ g/mL in PBS, overnight) and blocked with 0.5% gelatine in PBS (1 h). After washing, appropriately diluted samples and standard were added and



incubated for 3 h. Thereafter, peroxidase-conjugated anti-rat Ig (Dako Cytomation, Glostrup, Denmark) was applied for 2 h for total anti-OVA Ab quantification, while biotin-conjugated anti-rat IgG1, IgG2a, IgG2b or IgG2c (BD Biosciences, San Diego, CA, USA) was added to assess the concentration of anti-OVA Abs from these isotypes. In this case, an additional incubation with peroxidase-conjugated ExtrAvidin (Sigma-Aldrich, Madrid, Spain) was needed. Bound peroxidase was evinced using H₂O₂ and a solution of o-phenylenediamine dihydrochloride as the chromogenic substrate. Absorbance was measured at 492 nm using a microplate photometer (Labsystems Multiskan, Helsinki, Finland), and results were expressed as arbitrary units (A.U.) relative to a standard constituted by a pool of OVA-immunized rat plasmas from a previous study (43), assigned a value of 800 A.U./mL for total anti-OVA Abs and 100 A.U./mL for each anti-OVA IgG isotype.

2.9. Statistical analysis

Statistical analysis was conducted using SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA). Prior to the main analysis, potential effects of sex and immunization status were assessed to determine whether data could be pooled. If no significant differences were observed between sexes, data were combined for further analysis. Normality and homogeneity of variances were evaluated using the Shapiro–Wilk and Levene tests, respectively. When data followed a normal distribution with homogeneous variance, a one-way ANOVA was performed, followed by Tukey's post-hoc test for multiple comparisons. In cases where these assumptions were not met, the Kruskal–Wallis test was applied to assess significant differences ($p < 0.05$).

Additionally, nonmetric multidimensional scaling (NMDS) was employed in RStudio (version 4.4.2, R Foundation for Statistical Computing, Vienna, Austria) using the 'vegan' package to identify clusters of sample similarities based on immune factor composition. The 'envfit' function was used to assess the association of factors with sample ordination in the NMDS plot. Statistical significance was defined as $p < 0.05$.

The effect of the maternal diet and OVA immunization was evaluated in offspring lymphocytes from the spleen, MLNs and thymus and Ig from plasma, MLNs and GW. In all cases, results for males and females in the same group did not differ, thus data were gathered.

3. Results

3.1. Morphometric variables

The animals' growth was monitored daily from day 1 until the last day of the study (week 11 of their life). The pups weighed between 5 and 7 g at birth and about 59–60 g at the end of the suckling period (week 3). By the end of the study, female rats weighed between 232 and 261 g, and males between 345 and 382 g. No differences in body weight during the study were observed due to the maternal diet or due to immunization at week 7 of life (Figure 1).

At the end of the study, BMI and Lee index were calculated and organ weights were recorded, with most showing no differences between groups (Supplementary Table 1).

3.2. Lymphoid composition of spleen, mesenteric lymph nodes and thymus

With regard to the spleen, no significant differences were observed in the proportions of TCR $\alpha\beta$ +, TCR $\gamma\delta$ +, NK, and NKT cells of the offspring due to the maternal FP diet or OVA immunization (Table 2). Nevertheless, B cell proportion increased in REF OVA animals respect to the nonimmunized REF group. Within TCR $\alpha\beta$ + lymphocytes, the proportions of Th (CD4+) and Tc (CD8+) cells changed in the OVA group with the maternal FP diet, inducing a relatively higher CD8+ cell proportion and, consequently, a lower CD4+ cell percentage and CD4/CD8 ratio than in the REF OVA group. Similar results were found for the CD4+ and CD8+ subsets in the NKT population.

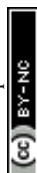
When considering CD8+ cells in NK lymphocytes, the percentage decreased in both OVA-immunized groups with respect to nonimmunized counterparts (Table 2).

With respect to the MLNs, the results are quite similar to those obtained for the spleen. Thus, no significant differences were found in the major lymphocyte populations, including B cells, TCR $\alpha\beta$ +, NK, and NKT cells, among the experimental groups (Table 3). However, the immunization led to a lower percentage of TCR $\gamma\delta$ + cells in the REF OVA group than in the nonimmunized group (REF group). Furthermore, as also noted in the spleen, the CD8+ cell proportion within the TCR $\alpha\beta$ + population was higher and the CD4/CD8 ratio was lower in the FP OVA-immunized group than in the REF OVA-immunized group. Similarly, in NKT cells, OVA immunization caused a relatively higher proportion of CD8+ cells (and reciprocally a lower CD4+ cell percentage) in animals from FP mothers (FP OVA group vs. FP group).

In NK cells, the percentage of CD8+ cells was lower due to the maternal FP diet in nonimmunized groups (FP group vs. REF group) and it was also lower after OVA immunization in animals from REF mothers (REF OVA group vs. REF group).

The proportion of CD103+ cells was also assessed in the T cell subsets of spleen and MLN cells. No changes were observed in CD103+ cells within T CD4+ and T CD8+ cell populations in the spleen (Figure 2). In contrast, in the MLNs, OVA immunization in REF animals (REF OVA group) led to an increase in CD103+ cell proportions in T CD4+ and T CD8+ lymphocytes. However, this increase was not observed in the offspring of mothers fed the FP diet.

In the thymus, T cell maturation was assessed by analysing the proportions of CD4+ and CD8+ subsets (Supplementary Figure 1). No differences were observed between groups in the proportions of cells with a different degree of maturation, i.e., CD4-CD8- (DN), CD4+CD8+ (DP), CD4+CD8- (CD4+ SP), and CD4-CD8+ (CD8+ SP) thymocytes. Within the DN subset, the proportion of CD25+ and CD44+ cells was also measured (Figure 3). The proportion of DN1 cells (CD44+CD25-) was reduced in OVA-immunized FP animals compared to the nonimmunized FP group, whereas DN4 cell



percentage (CD44-CD25-) was reciprocally increased. No significant changes were found in DN2 cell proportion (CD44+CD25+) and DN3 cell proportion (CD44-CD25-) due to the maternal diet or OVA immunization.

3.3. Immunoglobulin concentration in plasma and mesenteric lymph nodes

To explore the effect of an FP maternal diet and OVA immunization on the overall systemic humoral response, we first measured plasma concentrations of IgG, IgM, and IgA, as well as IgG isotypes (IgG1, IgG2a, IgG2b, IgG2c) (Figures 4a – 4c).

In plasma, there was a significant increase in total IgG concentrations in both REF and FP OVA-immunized groups compared to their respective nonimmunized groups (Figure 4a). This increase was due to elevated levels of all IgG subclasses (Figure 4b), and was less pronounced in the FP OVA group, mainly due to lower concentrations of IgG2b and IgG2c compared to the REF OVA group. Given that, in rats, IgG2b and IgG2c are associated with a Th1-type response, and IgG1 and IgG2a with a Th2-type response (43–46), we calculated the ratio of IgG2b+IgG2c to IgG1+IgG2a concentrations. The results indicated a lower ratio in the FP OVA group (Figure 4c).

As regards plasma IgM and IgA concentrations, higher levels of IgM in FP OVA animals than in FP nonimmunized rats were observed, while IgA increased in the REF OVA group with respect to the REF group but not in those animals receiving the FP diet (Figure 4a).

A similar pattern was observed in the mucosal compartment estimated by Ig levels in MLNs, with both the REF OVA and FP OVA groups exhibiting a higher total IgG concentration than to their nonimmunized counterparts (Figure 4d), due to increased concentrations of IgG1, and IgG2a in both groups, and IgG2b and IgG2c just in the REF OVA group (Figure 4e). Similarly to plasma, the ratio between Ig associated with Th1/Th2 immune responses also revealed a lower Th1/Th2 ratio in FP OVA animals in this compartment (Figure 4f).

On the other hand, the low levels of IgM in MLNs did not change in response to the maternal diet or immunization, but the IgA concentration increased after immunization in those animals with the maternal FP diet with respect to the nonimmunized FP group (Figure 4d).

In line with the above findings, the NMDS ordination analysis based on Ig concentrations in plasma and MLNs further supported the presence of group-specific Ig profiles. Pairwise PERMANOVA comparisons indicated no significant difference between REF and FP non-immunized groups ($p = 0.18$), in line with their overlapping ordination. In contrast, the comparison between the REF OVA and FP OVA groups revealed a clear and statistically significant separation ($p = 0.01$). Likewise, REF animals immunized with OVA clustered apart from their non-immunized counterparts (REF vs. REF OVA, $p = 0.001$), and a similar pattern was observed within the FP background (FP vs. FP OVA, $p = 0.04$), indicating a clear impact of immunization on the overall Ig profile.

3.4. Humoral and cellular systemic immune response against OVA

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To assess the impact of the maternal diet in their adult progeny on the OVA-specific humoral immune response, total anti-OVA Abs were measured in offspring plasma over a period of 4 weeks (Figures 5a and 5b).

Immunized rats developed anti-OVA Abs that were detected after 2 weeks of immunization and increased progressively until the end of the study (Figure 5a). Interestingly, immunized offspring born from mothers fed the FP diet (FP OVA) exhibited consistently higher concentrations of anti-OVA Abs than the REF immunized group (REF OVA). This enhanced humoral response in the FP group was confirmed by a significantly greater AUC of the overall Ab response pattern (Figure 5b). In addition, anti-OVA IgG isotypes were quantified in plasma at the final time point (Figure 5c). Notably, the concentrations of IgG1 and IgG2a were higher in the FP OVA group than in the REF OVA group.

To assess the specific cellular response against OVA, a DTH reaction to OVA was carried out (Figures 5d and 5e). Both immunized groups (REF OVA and FP OVA groups) showed a significant DTH response at the OVA-injected ear, with significantly increased ear thickness and section weight compared to their respective PBS-injected control ears and to nonimmunized siblings. In this case, no significant influence of the maternal diet was observed in the DTH response in the adult offspring.

3.5. Mucosal Ab response against OVA

In addition to systemic responses, anti-OVA Ab levels were also assessed at the mucosal level at the final time point. Specifically, anti-OVA Ab concentration was measured in MLNs and GW (Figures 6a and 6b). Similarly to the plasma findings, offspring from the FP OVA group exhibited higher anti-OVA Ab levels in both MLNs and GW than those from the REF OVA group. In line with these findings, the NMDS ordination analysis based on anti-OVA Ab concentrations (Figure 6c) demonstrated a clear separation between the REF OVA and FP OVA groups. This separation was statistically significant (PERMANOVA, $p = 0.0001$), indicating that the overall Ab response profiles differed markedly between groups.

4. Discussion

This study addresses the need to understand the influence of the maternal diet during pregestation, gestation, and lactation on the long-term immune system function of offspring, in particular in shaping the specific immune response against an antigenic challenge in adult offspring. Here we demonstrate that a maternal diet enriched in fibre and polyphenols (FP) during that perinatal period, is able to enhance the immune response to an external challenge with ovalbumin (OVA) in adult offspring, highlighting the immunoprogamming effect of this particular diet.

Enriching the maternal diet with fibre —specifically inulin and pectin— and polyphenols, including quercetin, naringenin, hesperidin, catechin, and epicatechin, was chosen for two reasons. First, these components reflect the composition of a healthy dietary



pattern in a gestating/lactating Spanish Mediterranean cohort (35), ensuring physiological relevance and translational validity. Second, both dietary fibre and flavonoids, administered independently, have been documented as providing immunomodulatory benefits in both the mother and the offspring (47,48). For example, a GOS/inulin mixture administered during gestation and lactation in mice reduced IgE levels and clinical allergic responses in the offspring, promoting a more regulatory immune environment (47). Similarly, exposure to quercetin during gestation and lactation has been shown to modulate maternal–foetal interface immune cells, reducing inflammation, supporting T-cell homeostasis, and maintaining immune tolerance (48). Moreover, it has been stated that the direct intake of flavonoids can modulate the immune system by influencing immune cell activation, cytokine production, and Ab responses (49). Nevertheless, the use of dietary fibre and polyphenols together in dams and the effect on their offspring later in adulthood, particularly in the acquired immune response, has been not established before.

We have quantified the amount of specific anti-OVA Abs developed after 4 weeks of a single immunization. The offspring from FP-fed mothers exhibited higher levels of anti-OVA Abs in plasma than offspring from REF-fed mothers. This enhanced immunity in adult offspring, despite no direct exposure to the FP diet, indicates that the maternal diet enriched in fibre and polyphenols, beginning before pregnancy and maintained throughout the entire pregnancy and lactation periods, caused an immunological programming in the offspring. To the best of our knowledge, this is the first evidence indicating that such a diet can exert this type of long-term immunoprogramming effect. These findings, which demonstrate the potential of the maternal diet, are in line with the sharp contrast observed with those from maternal Western-style diet (WSD) interventions [reviewed in (50)].

The isotype of anti-OVA Abs was established at the end of the study. The levels of specific anti-OVA Abs belonging to IgG1 and IgG2a were markedly higher in all OVA-immunized animals compared to Abs belonging to IgG2b and IgG2c isotypes, which were scarcely detectable. This can be attributed to the use of alum as an adjuvant in the immunization, as it preferentially promotes a Th2-type immune response (51), which is usually responsible for fighting against parasites and extracellular infections (52,53). We found that the offspring born from FP-fed mothers exhibited even higher levels of anti-OVA IgG1 and IgG2a than immunized animals from REF-fed mothers. These findings are consistent with a study showing that intranasal immunization of mice with OVA plus polymerized caffeic acid, ferulic acid or p-coumaric acid elevated OVA-specific plasma IgG, particularly IgG1, showing the reinforcement of the immune response (54). In summary, a maternal diet enriched in fibre and polyphenols fed before gestation and maintained until the end of the lactation period, enhances the specific humoral immune response boost with an adjuvant that stimulates Th2 immune responses. It remains to know whether such a maternal diet is also able to modulate the Th1 immune responses using a suitable and specific adjuvant. Despite the enhanced specific humoral immunity, no differences were observed in the cellular response against OVA established by the DTH response depending on the maternal diet. This is consistent with previous reports indicating that the maternal

diet tends to have a limited or modest impact on cellular immune responses such as DTH in both animal models and human populations (55,56).

Besides the higher levels of specific anti-OVA Abs quantified in the plasma of animals born from mothers consuming an FP diet, we also found enhanced levels of anti-OVA Abs in the MLNs and in the GW from the same animals. These findings indicate that the enhancement in the immune response to OVA also affects the mucosal compartment, in particular, the gut-associated lymphoid tissue (GALT). This compartmental analysis was further supported by NMDS ordination, which revealed a clear clustering of anti-OVA Ab profiles across plasma, MLN, and GW, highlighting the coordinated enhancement of systemic and mucosal humoral responses induced by the maternal FP diet. Due to the connection of the GALT to other mucosal-associated lymphoid tissues (57) such as those present in the respiratory tract, boosting the immune system through the maternal diet could be very important and could have significant implications for fighting against the most common infections. Further studies must be focused in establishing the effect of FP maternal diet on a respiratory infection to demonstrate the potential of such diet also at this level. The results obtained here are in line with studies that reported enhanced mucosal Ab responses in neonates after maternal fibre supplementation (58,59). However, no studies have focused on the effect of a maternal diet enriched in polyphenols in the mucosal immune system of the offspring.

In addition to the enhanced specific Ab response in systemic and mucosal compartments in the FP group. OVA immunization in both FP and REF groups led to an increase in the plasma and MLN concentrations of IgG1 and IgG2a. However, the content of IgG2b, and IgG2c in plasma and MLN was increased only in the REF group. The IgG isotypes made it possible to estimate the ratio between Th1-related isotypes (i.e., IgG2b and IgG2c) and Th2-related isotypes (i.e., IgG1 and IgG2a) in rats (43). The resulting ratio allows to suggest that FP offspring had a Th2 enhanced immune response, which is consistent with the results concerning specific anti-OVA Abs in plasma and mucosal compartments and the Th2 promoting effect of the adjuvant used. Overall, the results obtained for specific and total Igs indicate that a maternal FP diet induces a more efficient immune response, which in this case is based in a higher Th2 immune response and conversely, modulating the Th1 immune response that promotes inflammatory response. Nevertheless, although this suggestion is in line with results obtained from a direct diet enriched in some polyphenols (60,61), as stated before, it remains to know what would happen using an immune challenge specific for a Th1 immune response.

The mechanisms involved in the immunoprogramming effect on the development of humoral immune response were not established here, but we can suggest some insights based on previous results at both preclinical and clinical level. On the one hand, the maternal diet influences the placental transfer of bioactive compounds, such as Ig and cytokines (62). Moreover, after birth, maternal diet affects milk immune composition (63,64), and therefore it can modulate the offspring's immune system development. Additionally, the immunoprogramming effect could be consequence of changes in the



breast milk microbiota that are transferred from mother to infant enhancing infant immunity among other functions as described (65). Lastly, progeny could undergo epigenetic changes due to maternal diet. It has been described that early life constitutes a “window of epigenetic plasticity”, during which direct or indirect dietary exposures can permanently alter gene expression patterns and influence the long-term immunity. Both DNA methylation and microRNA changes, among other mechanisms, have been ascribed to polyphenols and other dietary components (66). Once the impact of the maternal dietary intervention on the offspring immunity has been demonstrated, further studies will focus on the mechanisms involved

As well as the impact of the maternal diet on humoral immune response, its influence on thymus, as primary lymphoid tissue, in spleen, as secondary systemic lymphoid tissue, and in MLNs, as secondary mucosal lymphoid tissue was also established. The higher proportion of CD8+ cells within TCRαβ+ and NKT lymphocytes both in the spleen and in MLNs of FP OVA-immunized offspring are in line with those obtained in a murine model in which maternal supplementation of a strain of the probiotic *Lactobacillus rhamnosus* during gestation and lactation promoted the development of virus-specific CD8+ T cells in the offspring (67). On the other hand, we found no changes in the proportion of cells expressing CD103 in spleen T CD4+ and T CD8+ lymphocytes indicating that maternal diet did not influence on tissue-resident memory cells or regulatory T cells (68,69). Nevertheless, the proportion of CD103+ cells increased in MLNs after OVA immunization in REF animals but not in the FP group indicating that the maternal FP diet impact on these cells. Further studies must clarify the meaning of such effect.

In the thymus, the lymphocyte maturation from double negative or DN cells (with DN1-DN4 stages), to double positive or DP cells (CD4+CD8+ cells), and, finally, single positive or SP cells (CD4+ cells and CD8+ cells) remained unchanged by maternal diet. However, FP-OVA offspring showed a reduced proportion of DN1 cells and an increased percentage of DN4 cells with respect to their nonimmunized counterparts. This could indicate that OVA immunization in the offspring of FP mothers accelerates the thymocyte maturation in this particular early stage. Although more matured thymic cells, such as CD8+ SP cells, were not affected by the maternal diet or immunization, the increase in DN4 cells could indicate that the maternal diet influences early T-cell development, potentially biasing the peripheral immune profile toward a cytotoxic CD8+ phenotype as observed in the spleen and MLNs. Further studies focused in the function of CD8+ cells might confirm such hypothesis.

The influence of polyphenols and fibre present in the maternal diet on the lymphoid tissues of adult offspring could be attributed to the same mechanisms suggested before. Firstly, they could be derived from the presence of polyphenolic metabolites during gestation or lactation, through placenta or breast milk, respectively, as it has been described (62,70). In addition, the maternal FP diet impacted its microbiota composition and, consequently, that from the litter in early life, leading to a more effective immune development (71). Finally, both SCFA, derived from dietary fibre, and some polyphenols have epigenetic potential in terms of DNA acetylation/methylation

or miRNA modulation (71,72). Consequently, future studies should focus into establishing the precise mechanisms involved in the effect of the maternal diet on lymphoid tissues composition and function.

Finally, it is important to emphasize that a maternal diet enriched in fibre and polyphenols during pregestation, gestation, and lactation did not influence the growth of the offspring. Minor, sex-dependent changes in relative organ weights were observed, suggesting subtle physiological effects without impacting overall somatic growth. It is relevant to explore whether a maternal diet influences offspring body weight, as maternal obesity and nutritional status have been shown to impact metabolic and immune outcomes in the progeny. Maternal obesity can predispose offspring to altered immune responses and increased risk of metabolic disorders, highlighting the importance of maternal nutrition beyond immune programming (73).

5. Conclusions

This study demonstrates that maternal consumption of a diet enriched in fibre and polyphenols, from pregestation through lactation, enhances both systemic and mucosal humoral immune responses against an antigenic challenge in adult offspring. Our findings provide novel evidence that such maternal dietary patterns can induce long-term immunoprogramming effects, improving the capacity of offspring to mount effective specific immune responses.

Although mechanistic studies are needed, this research may contribute to understanding the role of maternal nutrition in the long-term modulation of immune function and provide insights into preventive strategies for immune-related diseases.

Author contributions

Sergi Casanova-Crespo: Writing – original draft, Methodology, Formal analysis. Daniela Ceballos-Sánchez: Investigation, Methodology. María José Rodríguez-Lagunas: Writing – review & editing, Investigation. Malén Massot-Cladera: Writing – review & editing, Investigation, Methodology. Margarida Castell: Writing – review & editing, Investigation, Supervision. Francisco José Pérez-Cano: Writing – review & editing, Investigation, Project administration, Funding acquisition, Conceptualization.

Conflicts of interest

There are no conflicts to declare.

Data availability

The datasets used and/or analysed during the current study are available with the corresponding author upon reasonable request.

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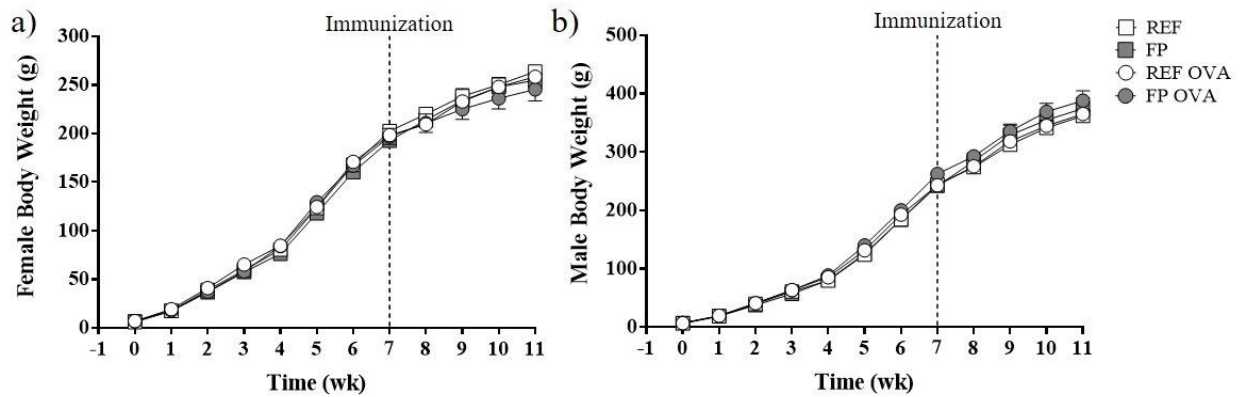


Figure 1. Body weight throughout 11 weeks in females (a) and males (b). Animals from dams fed the reference diet (REF) are represented in white symbols, and animals from dams fed the fibre- and polyphenol-enriched diet (FP) are represented in grey symbols. Nonimmunized groups are represented in squares and immunized groups in circles. The vertical dashed line indicates the day of immunization. Results are expressed as mean \pm SEM ($n = 3-9$).

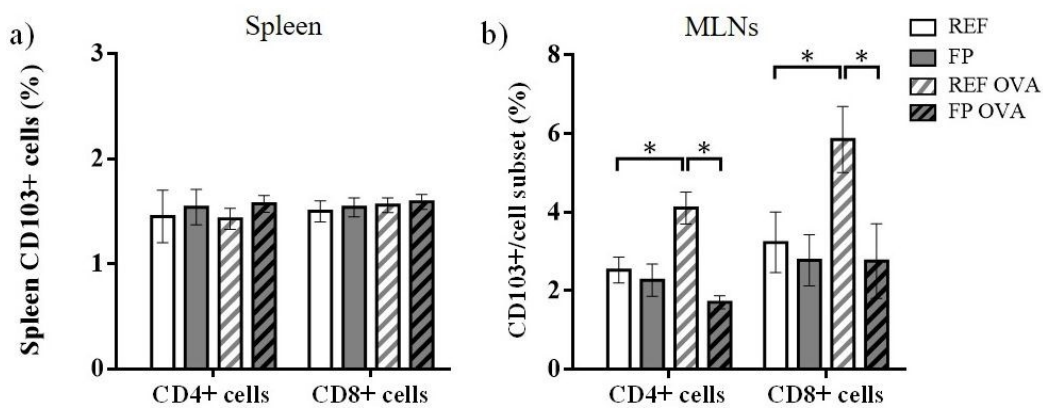


Figure 2. Percentage of CD103+ cells in CD4+ and CD8+ lymphocytes from the spleen (a) and MLNs (b). REF groups were those born from mothers fed the reference diet; FP groups comprised those born from mothers fed a fibre- and polyphenol-enriched diet; OVA groups consisted of those that were immunized at week 7 of life. Results are expressed as mean \pm SEM ($n = 7-12$). Statistical differences: * $p < 0.05$ vs. compared with the respective experimental group.



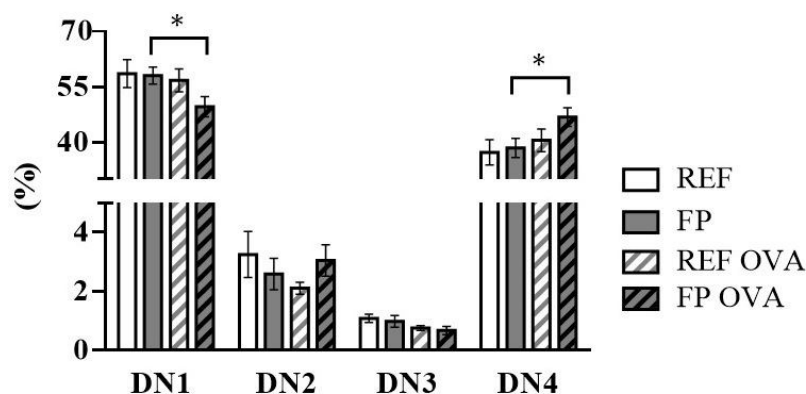
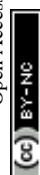
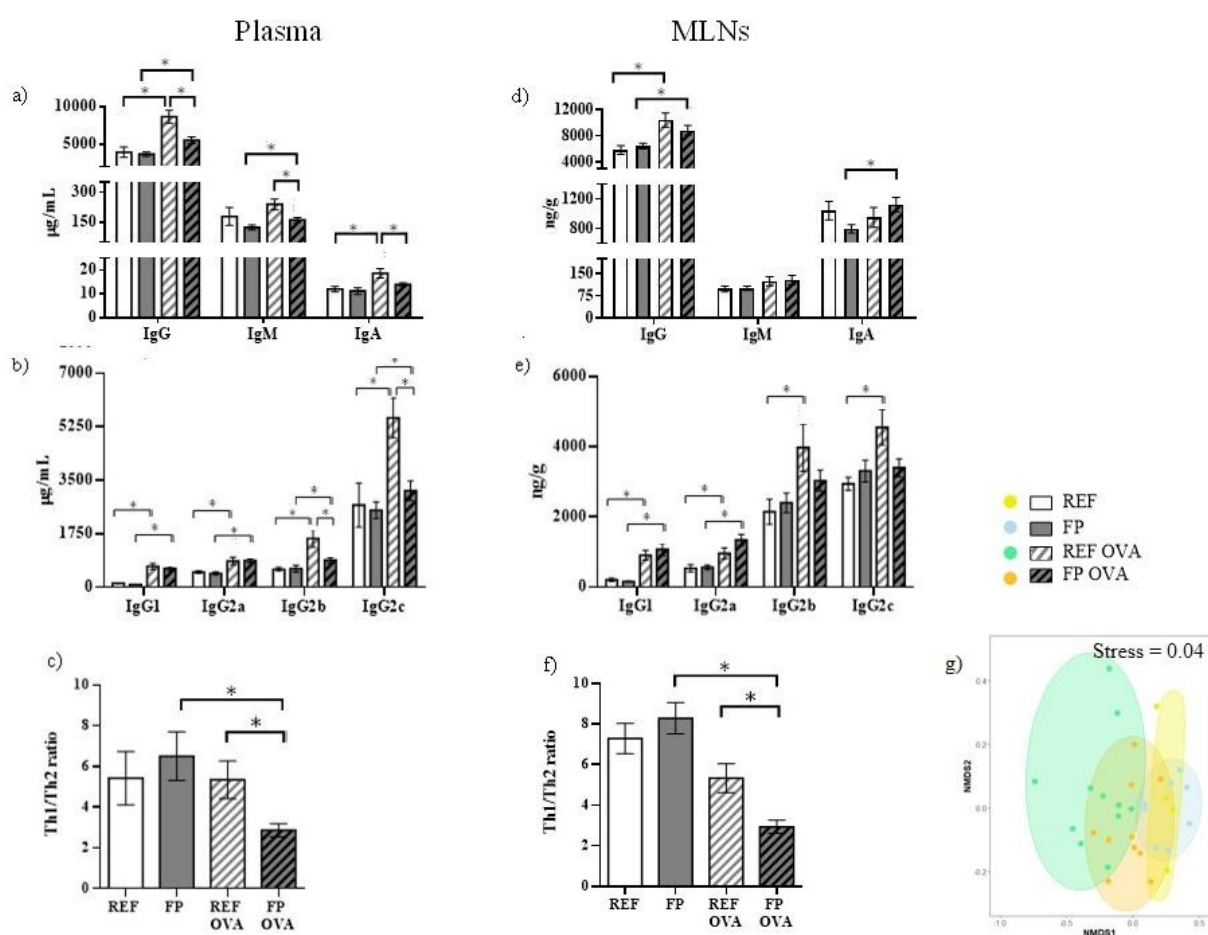


Figure 3. Percentage of double negative cells (DN or CD4-CD8-), DN1 cells (CD44+CD25-), DN2 cells (CD44+CD25+), DN3 cells (CD44-CD25+), and DN4 cells (CD44-CD25-) (a). REF groups were those born from mothers fed the reference diet; FP groups consisted of those born from mothers fed a fibre- and polyphenol-enriched diet; OVA groups comprised those that were immunized at week 7 of life. Results are expressed as mean \pm SEM (n = 7-12). Statistical differences: * p < 0.05 vs. compared with the respective experimental group.



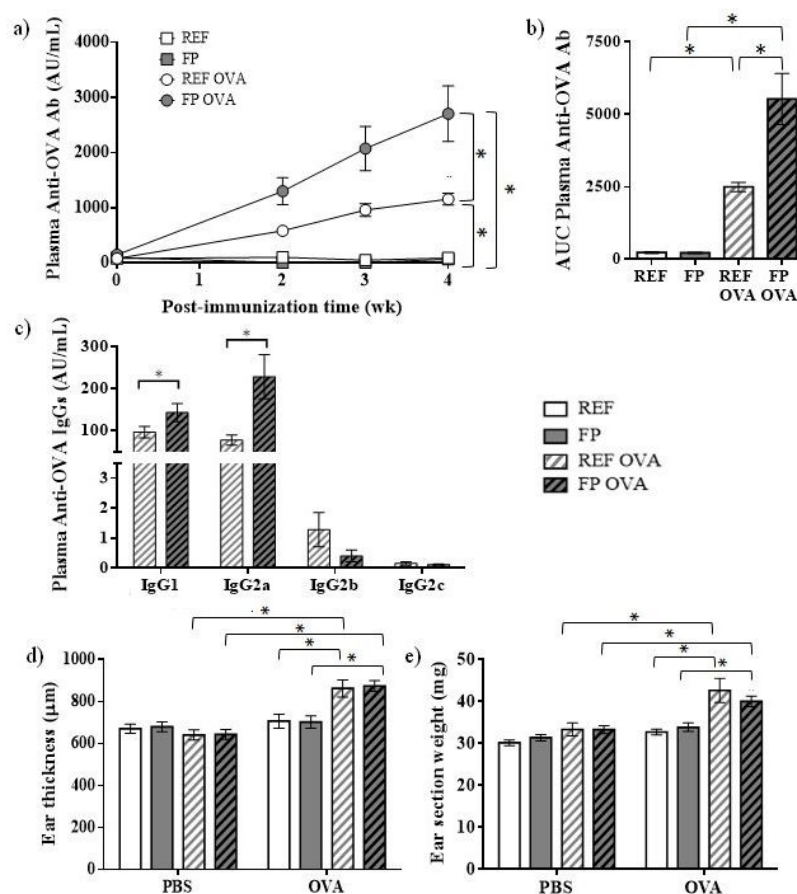


Figure 5. Time course of plasma anti-OVA Abs over time expressed in arbitrary units (AU/mL) (a) and as area under the curve (AUC) (b). Plasma anti-OVA Abs by IgG isotypes at end point (c). DTH response as ear thickness (μm) (d) and ear tissue weight (mg) (e) measured 24 h post-OVA challenge. REF groups consisted of those born from mothers fed the reference diet; FP groups were those born from mothers fed a fibre- and polyphenol-enriched diet; OVA groups comprised those that were immunized at week 7 of life. Results are expressed as mean \pm SEM (n = 7-12). Statistical differences: * p < 0.05 vs. compared with the respective experimental group.

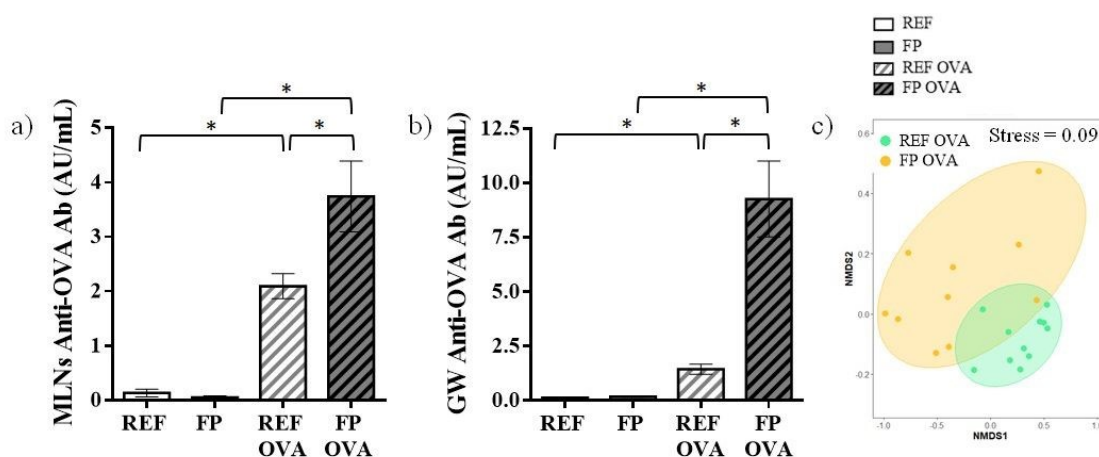


Figure 6. Total anti-OVA Abs in MLNs (a) and GW (b). Non-metric multidimensional scaling (NMDS) ordination of anti-OVA Ab concentration measured in plasma, GW, and MLNs (c) (Bray-Curtis dissimilarity). REF groups comprised those born from mothers fed the reference diet; FP groups consisted of those born from mothers fed a fibre- and polyphenol-enriched diet; OVA groups were those that were immunized at week 7 of life. Results are expressed as mean \pm SEM (n = 7-12). Statistical differences: * p < 0.05 vs. compared with the respective experimental group.



Table 1. Composition of the experimental diets used in the study.View Article Online
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Components	REF Diet (g/kg)	FP Diet (g/kg)
Casein	200	200
L-Cysteine	3	3
Cornstarch Flour	379.186	289.186
Inulin	0	80
Pectin	0	10
Maltodextrin	132	132
Sucrose	100	100
Cellulose	50	50
Soybean Oil	70	70
Mineral Mix (TD94049)	48	48
Ferric Citrate	0.3	0.3
Vitamin Mix (TD94047)	15	15
Choline Bitartrate	2.5	2.5
Tertiary Butylhydroquinone	0.014	0.014
Polyphenols	0	5
Catechin	0	1
Epicatechin	0	1
Hesperidin	0	1.5
Naringenin	0	0.75
Quercetin	0	0.75



Table 2. Lymphocyte composition (%) in the spleen.View Article Online
DOI: 10.1039/D5FO04563K

	REF	FP	REF OVA	FP OVA
Main lymphocyte populations				
B cells (CD45RA+)	31.03 ± 1.80	34.11 ± 2.59	40.15 ± 2.14#	40.77 ± 3.96
TCRαβ+ cells (TCRαβ+ NK-)	42.10 ± 1.25	39.55 ± 1.98	37.79 ± 1.44	37.13 ± 2.49
TCRγδ+ cells	3.29 ± 0.31	3.05 ± 0.16	2.46 ± 0.15	2.42 ± 0.17
NKT cells (TCRαβ+ NK+)	13.85 ± 1.02	13.46 ± 0.85	11.69 ± 0.56	11.83 ± 1.31
NK cells (TCRαβ- NK+)	9.69 ± 0.94	10.53 ± 1.23	8.39 ± 0.56	7.92 ± 0.60
Subsets within particular cells				
CD4+ in TCRαβ+ cells	68.45 ± 1.55	67.98 ± 1.11	72.01 ± 1.14	66.95 ± 1.44*
CD8+ in TCRαβ+ cells	29.78 ± 1.60	29.23 ± 1.70	26.00 ± 1.11	31.02 ± 1.35*
ratio CD4 / CD8 in TCRαβ+ cells	2.33 ± 0.17	2.45 ± 0.12	2.84 ± 0.17	2.21 ± 0.14*
CD8+ in TCRγδ+ cells	91.12 ± 1.05	90.94 ± 0.67	91.44 ± 0.58	91.46 ± 0.81
CD4+ in NKT cells	47.00 ± 0.96	47.95 ± 1.40	51.25 ± 1.39	45.16 ± 1.84*
CD8+ in NKT cells	50.20 ± 1.04	49.35 ± 1.34	46.17 ± 1.31	52.02 ± 2.11*
CD8+ in NK cells	37.38 ± 1.98	38.50 ± 1.83	29.97 ± 2.60#	27.27 ± 2.57#

The percentages of the main lymphocyte populations are expressed as cell proportions within the total lymphocyte gate, whereas the percentages of subsets are expressed as cell proportions within the particular indicated cells. Results are expressed as a percentage and mean ± SEM (n = 7-12). REF groups consisted of those born from mothers fed the reference diet; FP groups comprised those born from mothers fed a fiber- and polyphenol-enriched diet; OVA groups were those that were immunized at week 7 of life. Statistical differences: * p < 0.05 vs. reference diet in the same immunized condition; # p < 0.05 vs. not immunized in the same diet condition.



Table 3. Lymphocyte composition (%) in the MLNs.View Article Online
DOI: 10.1039/D5FO04563K

	REF	FP	REF OVA	FP OVA
Main lymphocyte populations				
B cells (CD45RA+)	24.73 ± 1.08	28.63 ± 2.63	28.86 ± 1.93	30.70 ± 3.14
TCRαβ+ cells (TCRαβ+ NK-)	67.93 ± 2.01	63.54 ± 2.91	62.51 ± 1.92	61.07 ± 3.00
TCRγδ+ cells	1.70 ± 0.12	1.44 ± 0.13	1.21 ± 0.10 [#]	1.26 ± 0.05
NKT cells (TCRαβ+ NK+)	5.01 ± 0.84	6.06 ± 0.50	6.87 ± 0.42	6.50 ± 0.76
NK cells (TCRαβ- NK+)	2.34 ± 0.59	2.99 ± 0.40	2.89 ± 0.45	2.84 ± 0.36
Subsets within particular cells				
CD4+ in TCRαβ+ cells	71.50 ± 1.04	72.44 ± 0.83	72.87 ± 0.96	70.79 ± 1.16
CD8+ in TCRαβ+ cells	26.90 ± 0.89	26.05 ± 0.83	24.18 ± 0.97	27.60 ± 1.09*
ratio CD4 / CD8 in TCRαβ+ cells	2.67 ± 0.13	2.81 ± 0.12	3.11 ± 0.17	2.60 ± 0.13*
CD8+ in TCRγδ+ cells	93.24 ± 0.52	92.59 ± 1.32	93.20 ± 0.62	93.36 ± 0.66
CD4+ in NKT cells	64.55 ± 1.29	59.80 ± 1.60	58.38 ± 2.28	55.70 ± 2.38 [#]
CD8+ in NKT cells	36.38 ± 1.43	32.53 ± 1.32	38.79 ± 2.31	41.31 ± 2.11 [#]
CD8+ in NK cells	15.68 ± 1.00	10.62 ± 0.74*	9.60 ± 0.92 [#]	10.43 ± 0.94

The percentages of the main lymphocyte populations are expressed as cell proportions within the total lymphocyte gate, whereas the percentages of subsets are expressed as cell proportions within the particular indicated cells. Results are expressed as a percentage and mean ± SEM (n = 7-12). REF groups consisted of those born from mothers fed the reference diet; FP groups comprised those born from mothers fed a fiber- and polyphenol-enriched diet; OVA groups were those that were immunized at week 7 of life. Statistical differences: * p < 0.05 vs. reference diet in the same immunized condition; # p < 0.05 vs. not immunized in the same diet condition.



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Data availability

The data that support the findings of this study are available from the corresponding author, MC, upon reasonable request.

