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Low-dose perinatal supplementation with *Enterococcus faecalis* increases concentrations of short-chain fatty acids in the offspring but does not protect against allergic asthma

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Abstract

Childhood allergic asthma is associated with a dysbiotic gut microbiome in early life, and maternal perinatal treatment with probiotics is a potential way alter the infant microbiome, which may improve asthma outcomes. This study used a mouse model to examine the effect of maternal supplementation with the probiotic *Enterococcus faecalis* on faecal short-chain fatty acid (SCFA) concentrations and asthma risk in the offspring. Pregnant/lactating mice were treated daily, from gestation day 6 to postnatal day 21, with an oral suspension of 10^6, 10^7 or 10^8 colony-forming units of a live preparation of the probiotic *E. faecalis* (Symbioflor®1). At weaning, offspring were subjected to an ovalbumin-induced experimental asthma protocol. Faeces were collected from the mothers and offspring at several different time points to determine SCFA concentrations. It was found that maternal supplementation with *E. faecalis* did not alter litter size, sex ratio or offspring weight, and was associated with an increase in SCFAs in offspring faeces at weaning and after allergy induction. However, allergic offspring from *E. faecalis* supplemented mothers showed no difference in asthma severity when compared with allergic offspring from control mothers. In conclusion, although maternal perinatal supplementation with low-dose *E. faecalis* was associated with increased faecal SCFAs in the offspring, it did not protect against offspring asthma. This is may be because SCFA concentrations were not increased to an immunoprotective level. We recommend that future studies concentrate on probiotic supplementation in high-risk cases, for instance, to repair gut dysbiosis resulting from antibiotic use in pregnant mothers or their infants.

Keywords: asthma, developmental origins of health and disease, mouse model, pregnancy, probiotic

Introduction

Allergic asthma is a multifactorial disease that commonly develops in childhood and manifests as lung inflammation and hyperreactivity to normally harmless aeroallergens. The incidence of asthma has been steadily increasing in many countries, and evidence from both human and mouse studies indicates that the neonatal environment plays a role in this phenomenon, particularly with regard to the gut microbiome (1, 2). The early-life gut microbiota is essential for immune system maturation, and alterations to the infant commensal gut bacteria precede asthma development (3), resulting in a more severe asthma phenotype in both humans and mice (4–7). For these reasons, probiotic supplementation is regarded as a possible strategy for childhood asthma prevention. Although many human cohort studies have examined the effect of infant probiotic supplementation on asthma development (8–12), there is currently insufficient evidence to recommend this course of action (13, 14). This is likely due to a large heterogeneity between studies involving differences

in the probiotic strains used, as well as the timing and duration of treatment (14–18).

Considering effective timing for intervention, since the maternal and infant gut microbiota are strongly associated at birth, maternal probiotic supplementation may be a more effective solution for very early alteration of the infant microbiome. Nevertheless, studies examining maternal probiotic intervention during pregnancy are limited and conflicting. While pregnancy studies did not find an overall protective effect on childhood asthma development (17, 19), sub-analyses in several cohorts indicated positive effects according to the time of follow-up and the type of probiotic used. These include: reduction in children with a high hereditary risk of atopic sensitization (20), decreased risk of allergic disease after 10-year follow-up (21) and a positive effect of specific bacterial strains on asthma development (22). There is a clear need for large, randomized controlled trials to assess the effect of probiotic supplementation during pregnancy on asthma development in specific subpopulations of children.

Because of the highly heterogeneous and complex nature of human cohorts, mouse models are an excellent means to provide insight into future cohort design, particularly regarding the timing of supplementation and the probiotic strain(s) used. Analysis of both human and mouse studies indicates that *Enterococcus faecalis* is a promising probiotic for perinatal supplementation. Human intervention studies have demonstrated that *E. faecalis* supplementation in early childhood can reduce rhinosinusitis episodes (23) and mouse models have shown that supplementation with *E. faecalis* bacteria (24) as well as bacterial extracts (25) protected against experimental asthma in adult mice. Additionally, supplementation with *E. faecalis* has been shown to increase the production of immunomodulatory short-chain fatty acids (SCFAs) in humans (26). Although *E. faecalis* is a promising candidate for asthma prevention, the effect of its administration during pregnancy on offspring asthma susceptibility has not yet been tested. The objective of this research is to examine the effect of maternal oral supplementation of *E. faecalis* during the perinatal time period on the development of experimental allergic asthma in the offspring.

Methods

Experimental design

12-Week-old Balb/c mice were obtained from Janvier labs (Le Genest-Saint-Isle) and kept under specific pathogen free conditions with a 12 h day/12 h night cycle. Food and water were provided *ad libitum*. After mating, the presence of a vaginal plug was considered gestation day (G)0. Plugged mice were treated daily from G6 to postnatal day (PN)21 with an oral solution of 10^6, 10^7, or 10^8 colony-forming units (CFU) of a live preparation of the probiotic *E. faecalis* (Symbioflor®1) in 35 µl of excipient solution and 15 µl of Ora-Sweet (Paddock Laboratories) delivered via micropipette. Control mice received 35 µl of excipient solution mixed with 15 µl of Ora-Sweet (Fig. 1).

At PN21, female pups were weaned and immediately subjected to an experimental asthma protocol. Sensitization involved subcutaneous injection with 10 µg of ovalbumin (OVA) Grade VI (Sigma) in 200 µl phosphate-buffered saline (PBS) on at PN21, 28 and 35. This was followed by a daily 20 min challenge with 1% aerosolized OVA Grade V (Sigma) on PN47, 48 and 49. Twenty-four hours after the last challenge, the mice were sacrificed, and blood, bronchoalveolar lavage (BAL) and lungs were collected to assess the asthma phenotype (Fig. 1).

Blood collection and serum antibody measurement

Blood was collected from the retro-orbital sinus of terminally anesthetised mice, and centrifuged to separate the serum, which was aliquoted and stored at −80°C. Enzyme-linked immunosorbent assays (ELISAs) were performed to determine the concentration of total IgG1, OVA-specific IgG1 and OVA-specific IgE antibodies in the serum. For total IgG1 measurement, Nunc MaxiSorp plates (Sigma-Aldrich) were coated with 0.5 mg ml^{-1} of IgG1 antibody stock solution (BD Pharmingen) and incubated for 2 h at room temperature. Plates were washed, and serum samples were incubated at room temperature for 2 h, then at 4°C overnight. After washing, plates were incubated with biotinylated anti-mouse IgG1 (BD Biosciences) for 2 h at room temperature, followed by a 30 min, room temperature incubation with streptavidin peroxidase (Sigma). Plates were developed using BM Blue POD Substrate (Roche) and the reaction was stopped with 2M sulphuric acid (Merck). Colour development was read at 450 nm with a Tecan Infinite 200 PRO. ELISAs for OVA-specific IgG1 and OVA-specific IgE were performed as described previously (27).

BAL: cytospins and flow cytometry

After tracheostomy of euthanized mice, lungs were washed with 1 ml of PBS plus complete protease inhibitor cocktail (Roche) to obtain BAL. BAL samples were centrifuged at 350 × *g* for 10 min and supernatant was aliquoted and stored at −80°C. Cell pellets were resuspended in 1 ml of 1% bovine serum albumin (BSA) and this suspension was used for both cytospin preparation and flow cytometry analysis. Cytospins were prepared from 100 µl of cell suspension, stained with a Differential Quik Stain Kit (Polyscience) and white blood cells were counted by two independent researchers. For flow cytometry, cell pellets were stained with Zombie Aqua live dead staining (BioLegend) and after washing cells were resuspended in staining antibody mix consisting of: CD11b (PE-Cy7), CD11c (AF488), CD45 (AF700), CD64(APC), Ly6C (PB), Ly6G (PerCP) MHCII (BV650) (all from BioLegend) and SiglecF (PE—BD Biosciences). After washing, these samples were resuspended in FACS buffer. Precision count beads (BioLegend) were added and samples were acquired using a BD LSRFortessa™ X-20 Cell Analyzer.

Lung histology and inflammation quantification

Lungs were fixed with 1 ml of 4% formaldehyde and embedded in paraffin, then 4 mm sections were cut. Lung sections were quantified for eosinophils using modified Sirius Red staining and for mucous producing goblet cells using periodic acid-Schiff (PAS) staining. Quantification was

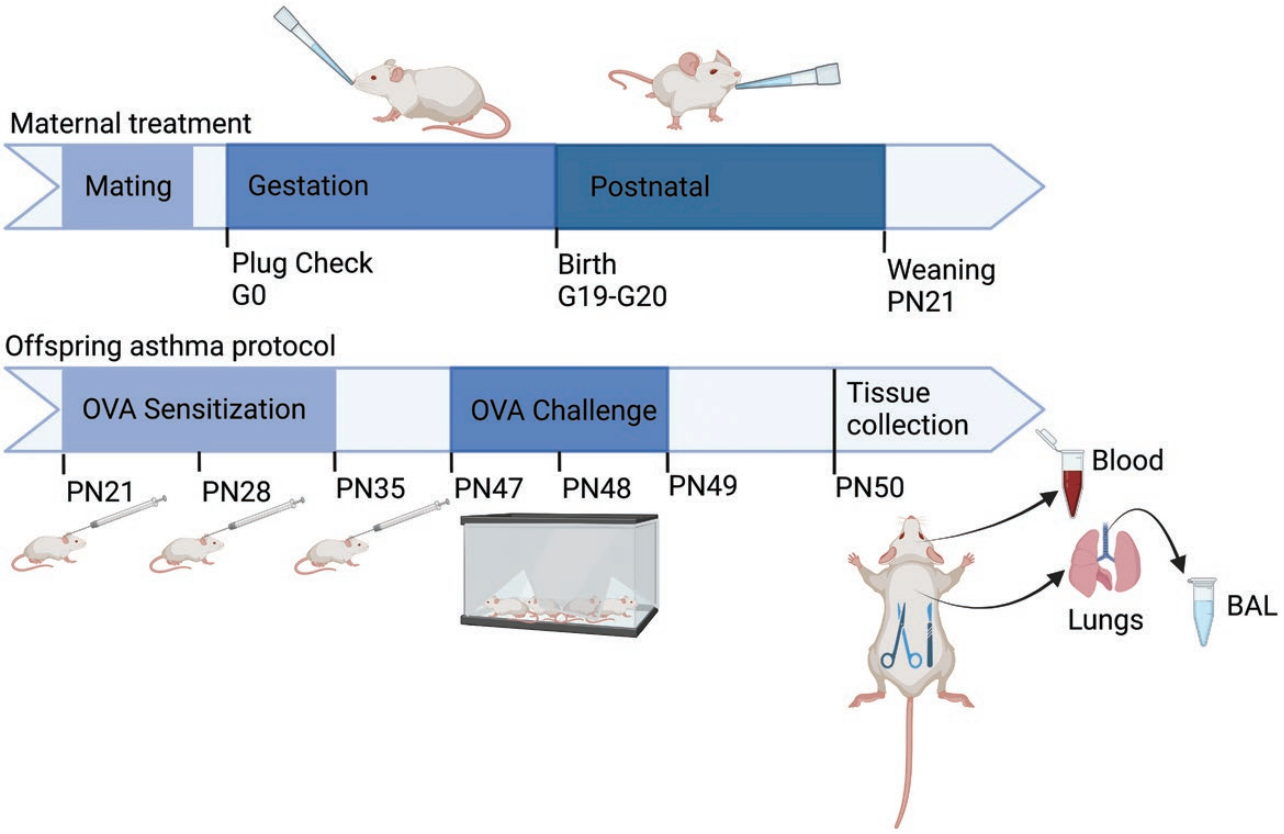

Fig. 1. Protocol for maternal perinatal treatment with *E. faecalis* and asthma induction in the offspring. After mating, pregnant mice were treated daily from G6 until PN21 with 10^6, 10^7, or 10^8 CFU of *E. faecalis* delivered orally in excipient solution and Ora-Sweet. Control dams received excipient solution and Ora-Sweet only. Starting at weaning (PN21) offspring were subcutaneously sensitized with OVA once per week for 3 weeks, followed 12 days later by three challenges with aerosolized OVA for 20 min once per day. At 50 days of age, offspring were sacrificed, and blood, BAL and lungs were collected for further analysis.

performed by a blinded pathologist by counting the stained cells in 10 high power fields.

Faecal E. faecalis quantification and SCFA analysis

Faeces was collected from mothers and offspring at the following time points: Mothers before mating (G0) and at weaning (PN21) and offspring at PN21 and after allergy induction at PN50. Concentrations of the SCFAs acetate, propionate, butyrate, valerate were analysed in the faeces using gas chromatography. Briefly, faecal lyophilizate was dissolved in 100 ml of 5M formic acid and 400 ml acetone, then centrifuged for 5 min at 4000 × *g*. SCFA concentrations were determined in the supernatant using a GC-2010 Plus gas chromatograph (Shimadzu) equipped with a flame ionization detection with a thin-film capillary column (Stabilwax-DA 30 m 0.25 mm 0.5 mm—Restek). The samples were spread out by split injection using the auto-sampler AOC-20s/I (Shimadzu). GCsolution Chromatography Data System (Shimadzu) was used for data processing. An external standard (Supelco WSFA-1 Mix—Sigma-Aldrich) was used for SCFA quantification.

Additionally, the *E. faecalis* concentration was measured in the faeces by qPCR. DNA was extracted using the QIAsymphony DSP Virus/Pathogen Mini-Kit (QIAGEN) according to the manufacturer's instructions. Automated isolation and pipetting of 96-well plates was performed using a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems) with the QIAsymphony SP/AS instrument (QIAGEN). To enumerate *E. faecalis*, the following primers were used: Efs 130F AAC CTA CCC ATC AGA GGG, Efs 490R GAC GTT CAG TTA CTA ACG. PCR amplification and detection was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems) in optical-grade 96-well plates. A standard curve was produced using the appropriate reference organism to quantify the qPCR values into copies per gram faeces. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was carried out following amplification to distinguish the targeted from non-targeted PCR products. Melting curves were obtained by slow heating at temperatures from 55 to 95°C at a rate of 0.2 C s^{-1}, with continuous fluorescence collection. The data were analysed using the ABI Prism software. Real-time PCRs were performed in triplicate.

Statistics

Normal distribution of samples was assessed using the Shapiro–Wilk normality test. Student's *t*-test or one-way ANOVA was used for parametric data analysis, whereas non-parametric data were analysed using Mann–Whitney

U or Kruskal–Wallis test. ANOVAs were performed with Dunnett's multiple comparisons test (comparing all groups against the control group). Data are presented as mean ± SEM. GraphPad Prism 9 software was used for all calculations.

Results

Perinatal supplementation with low-dose E. faecalis does not influence litter size, sex ratio or offspring weight

To assess how *E. faecalis* treatment influences pregnancy outcomes, we recorded litter size, as well as offspring weight and sex. The mean litter size was 4 pups and there were no differences in litter size or pup sex between the control and the treated groups (Fig. 2A). Measurement of offspring weight after allergy induction demonstrated no differences between the control and *E. faecalis* exposed offspring (Fig. 2B).

Maternal supplementation with low-dose E. faecalis is associated with increased SCFA production in the next generation

Since it is known that *E. faecalis* produces immunoregulatory SCFAs (26, 28) we next assessed SCFA concentrations in the faeces of control and *E. faecalis* treated mothers (10^8 CFU) and their offspring at the following time points: mothers before mating (G0), mothers and offspring at weaning (PN21) and offspring after allergy induction (PN50). As shown in Fig. 3A–D, although no differences in SCFA concentrations were observed between maternal control and treated groups at any time point, allergic offspring from treated mothers showed significantly increased concentrations of acetate, propionate, butyrate and valerate in their faeces. Butyrate was also significantly increased in offspring from *E. faecalis* treated mothers at weaning.

The observed increase in offspring SCFA concentrations led us to perform qPCR analysis of maternal and offspring faeces to determine *E. faecalis* concentrations. Surprisingly, our analysis, conducted at the same time points as the SCFA analysis, showed that *E. faecalis* was only detectable in a small portion of the measured samples (Fig. 3E).

Maternal treatment with low-dose E. faecalis during pregnancy and lactation does not influence offspring allergic asthma outcomes

To investigate whether oral administration of *E. faecalis* to mother mice during the perinatal period could influence asthma susceptibility, we assessed asthma development in allergic offspring from control and *E. faecalis* treated mothers. Differential cell counts in the BAL of allergic offspring showed no difference in the numbers of leukocytes, eosinophils, macrophages, or lymphocytes (Fig. 4A). An additional analysis by flow cytometry confirmed these results demonstrating no change in allergic offspring BAL Ly6G+ neutrophils, CD11b+CD11c-siglecF+ eosinophils, CD11b-CD11c+siglecF+ alveolar macrophages, or CD11bhiLy6C- macrophages between control and *E. faecalis* exposed groups (Fig. 4B). To further assess allergic asthma severity, we microscopically quantified eosinophils and mucus-producing goblet cells in allergic offspring lung tissue from control and *E. faecalis* treated mothers. As shown in Fig. 4C and D, quantitative histological analysis of MSR-stained eosinophils and PAS-stained goblet cells in 10 high power fields also showed no differences. Finally, to assess the possible systemic effects of maternal perinatal treatment with *E. faecalis* in the progeny, we measured serum antibody levels in allergic offspring. As shown in Figure 4E, no difference was observed in total IgG, OVA-specific IgG1 or OVA-specific IgE concentrations.

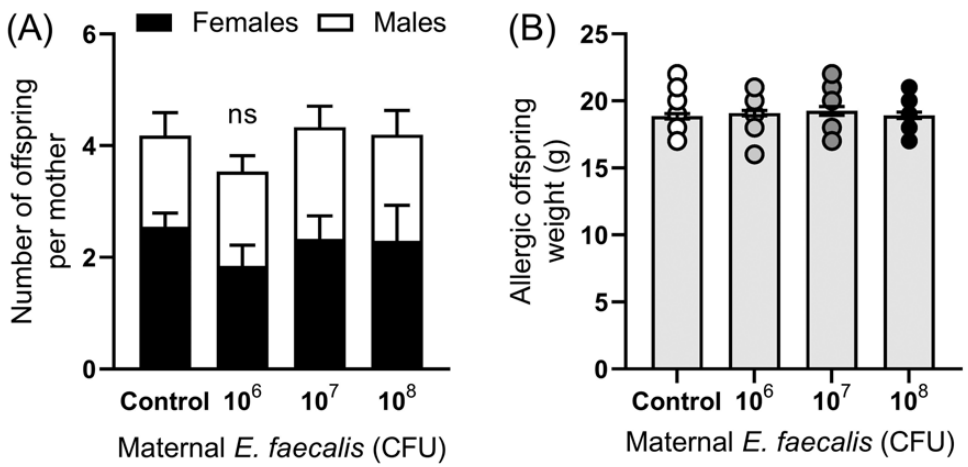

Fig. 2. (A) Litter size—total number of pups (from $n \geq 9$ mothers), separated into females and males born to control and *E. faecalis* treated mothers. (B) Weight of 7-week-old allergic offspring from control and *E. faecalis* exposed mothers (offspring $n \geq 20$). Means ± SEM are shown. Results represent two independently performed experiments. No comparisons were significant as per one-way ANOVA with Dunnett's multiple comparison test of 10^6, 10^7, and 10^8 groups compared with the control.

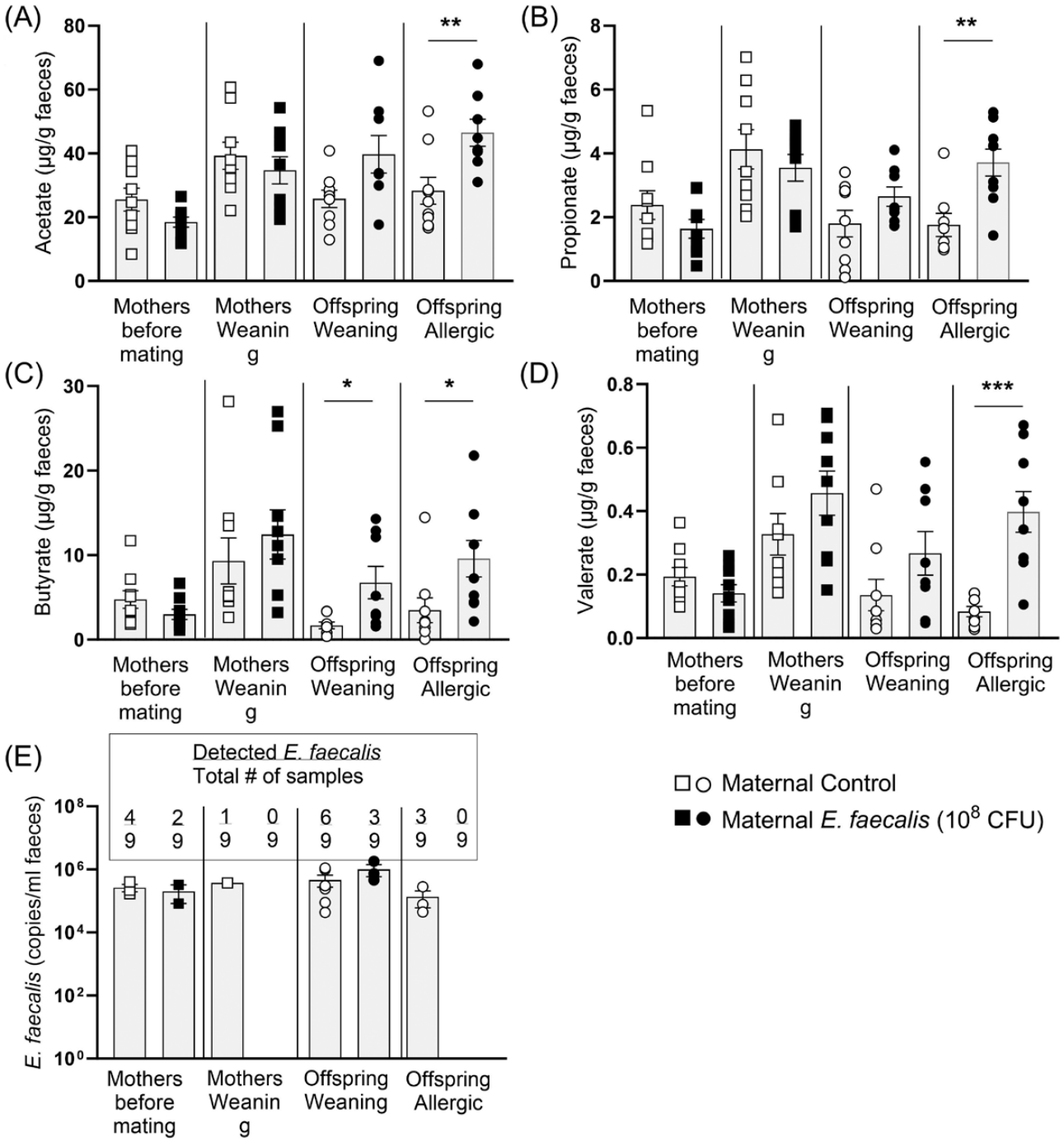

Fig. 3. Maternal *E. faecalis* treatment is associated with increased SCFAs in offspring faeces. (A–D) Acetate, propionate, butyrate and valerate. (E) Detection of *E. faecalis* in mother and offspring faeces by qPCR. Means ± SEM are shown. ($n = 9$) for all groups. Results represent two independently performed experiments. Significance is represented by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test or Mann–Whitney *U*-test of treated group versus control at each time point.

Discussion

Using a mouse model, we demonstrated that although maternal treatment with the probiotic bacteria *E. faecalis* during pregnancy and lactation increased faecal SCFA concentrations in the next generation, the observed increase was not enough to influence offspring allergic asthma severity. It is well known that high levels of SCFAs early childhood is associated with protection against asthma (29), and murine models demonstrate that direct supplementation with SCFAs also engenders protection (30). Considering pregnancy, a similar effect was shown in the progeny when pregnant mothers were given SCFA supplemented drinking water (31). In our study, though faecal SCFAs were increased in the probiotic supplemented group, there are

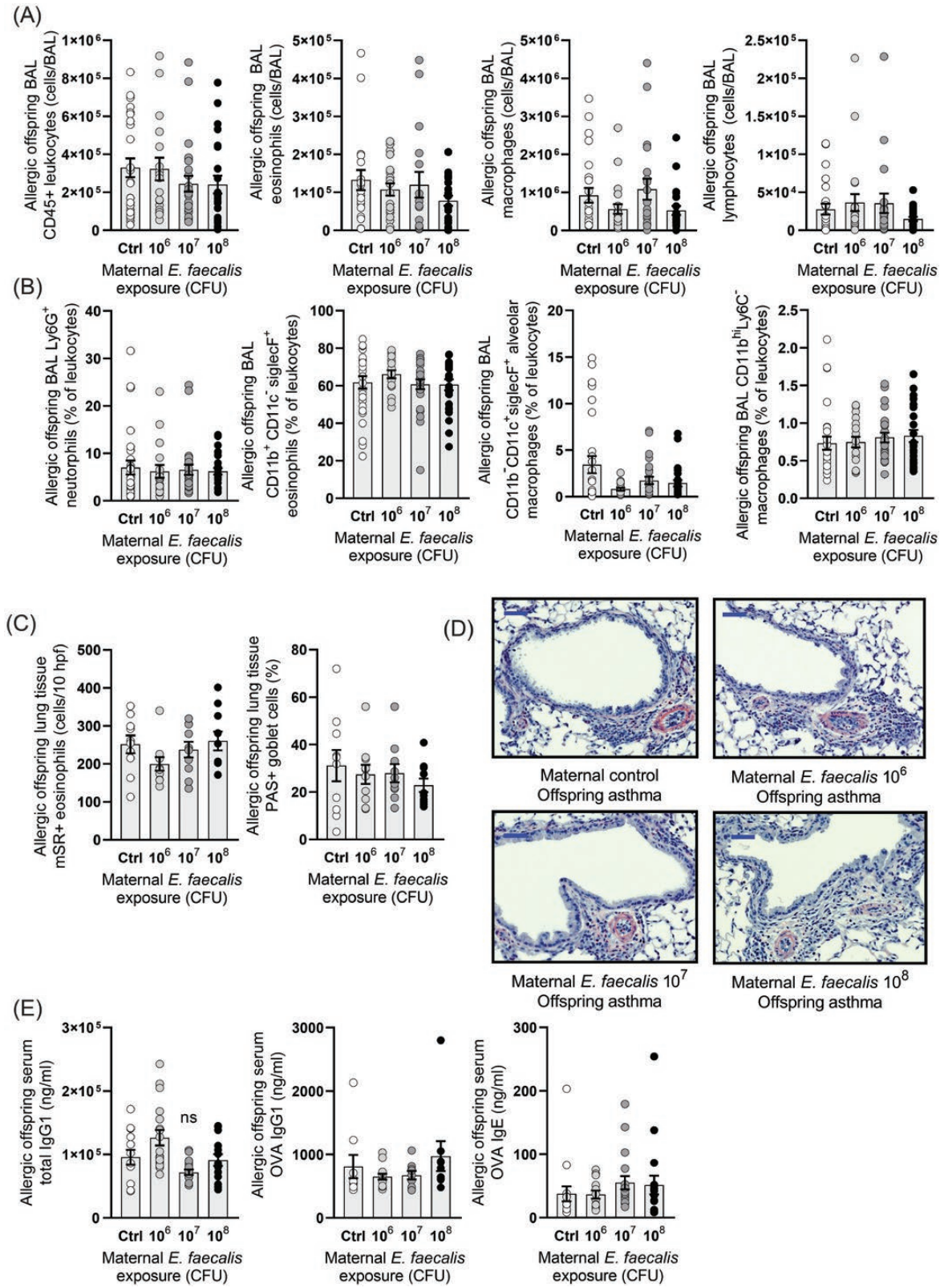

Fig. 4. Maternal perinatal treatment with *E. faecalis* did not alter allergic asthma severity in the offspring. (A) Differential cell counts of allergic offspring BAL: total leukocytes, eosinophils, macrophages and lymphocytes. (B) Flow cytometric analysis of allergic offspring BAL: Ly6G+ neutrophils, CD11b+CD11c-SiglecF+ eosinophils, CD11b-CD11c+SiglecF+ alveolar macrophages and CD11b^{hi}Ly6C-macrophages. (C) Quantification of Modified Sirius Red (MSR) stained lung tissue eosinophils and PAS-stained goblet cells counted in 10 high power fields. (D) Microscope images of MSR stained allergic offspring lungs. Scale bar = 100 μm. (E) Antibody concentrations of total IgG, OVA-specific IgG1 and OVA-specific IgE in the serum of allergic offspring. (A–E) Control (n = 28), *E. faecalis* 10^6 (n = 24), *E. faecalis* 10^7 (n = 20), *E. faecalis* 10^8 (n = 24). Means ± SEM are shown. Results represent two independently performed experiments. One-way ANOVA or Kruskal–Wallis test with Dunnett's multiple comparison test of each exposed group compared to the control. No comparisons were significant.

several reasons why they may not have contributed to protection against asthma. (i) The previously mentioned experiments used direct supplementation with SCFAs, which would arguably create a strong primary effect though the placenta and through the breast milk (32). Since the increased SCFAs in our experiment were a secondary result of the *E. faecalis* supplementation, it is reasonable to assume the effect may have been diluted compared to direct SCFA supplementation. (ii) Though the SCFAs in offspring faeces were increased, the concentrations may not have been high enough to have a physiological effect. After assessment of our study, we have several recommendations for the design of future animal models including treatment with viable bacteria, using higher probiotic concentrations and testing probiotics as a rescue strategy for antibiotic-induced gut dysbiosis in pregnant mothers.

In view of using live versus heat-killed bacterial or bacterial components, we propose that treatment with viable bacteria is an important consideration. Although treatment with bacterial components stimulates an anti-inflammatory immune response, live bacteria also stimulate distinct humoral immune responses, produce immunostimulatory metabolites such as SCFAs and interact with the intestinal microbiota (33–35). Supporting this, though previous studies have shown that treatment of adult mice with *E. faecalis* components was asthma protective (24), a recent epidemiological study assessing infant supplementation with a combination of heat-killed *E. faecalis* and *Escherichia coli* found no protective asthma effect in children (15). Bearing this in mind, we used live *E. faecalis* as a maternal perinatal treatment in our model. The product Symbioflor 1 was used, a probiotic available in the pharmacy which was shown to be protective against sinusitis (36) and lung disorders such as chronic bronchitis and COPD in humans (37).

Considering concentration-dependent effects, our model assessed three different Symbioflor concentrations for perinatal maternal treatment. Due to the use of this particular product, it was not possible to use concentrations higher than 1×10^8 CFU, which is a clear limitation of this study. However, we reasoned that since the foetus resides in a sterile environment until birth, the gut microbiome is initially seeded during the birthing process (38) and 1- to 7-day old neonatal mice have significantly lower alpha diversity in the gut than later stage neonates (39), that 10^6, 10^7 and 10^8 CFU would be acceptable concentrations for testing. As we were unable to consistently identify *E. faecalis* in the faeces of supplemented mothers and offspring, this indicates a need for significantly higher supplementation concentrations.

Finally, despite the fact that *E. faecalis* was either not found or only detectable in low copy number in the faeces of supplemented mothers and their offspring, we importantly still observed significantly increased concentrations of immunoprotective SCFAs in faeces. SCFAs are important mediators of intestinal homeostasis and their increased presence in the offspring faeces after maternal *E. faecalis* supplementation may be an indication of weak, but positive immunoprotective effects. If this is the case, we suggest the use of higher *E. faecalis* concentrations (10^{10}), and that perinatal probiotic supplementation might be better explored in high-risk cases, for example restoring gut microbiome homeostasis in mothers who have used antibiotics during pregnancy.

Conclusion

While our study cannot recommend maternal perinatal use of low-dose *E. faecalis* for protection against offspring asthma, a number of worthwhile conclusions can be drawn from this study. It may be preferable to use live probiotics for supplementation because of their ability to stimulate the production of SCFAs. Further, because of the potential immunoprotective effects of increased SCFA production, future research should examine the effect of maternal probiotic supplementation after antibiotic use, an event that can interfere with gut colonization in early life and thus increase the risk of asthma development.

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Conflict of interest statement: A.S. is an employee of MVZ Institut für Mikroökologie GmbH, a SME that produced the *E. faecalis* probiotic used for this study. M.L.C. received funding from MVZ Institut für Mikroökologie GmbH for the experiments conducted in this study. Other authors declare no competing financial interest.

Ethics approval

The animal experiments were approved by Berlin authorities (Ethics committee: Landesamt für Gesundheit und Soziales—LAGeSo G0209-17) and were performed according to German and international regulatory guidelines.

Author contributions

A.S. and M.L.C. designed the study and supervised the experiments. J.L.A., M.M.A., S.D. and F.G.K. performed the experiments and analysed the data. A.S. provided funding for the experiments. All authors were involved in manuscript writing and have approved the final version.

Data Availability

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

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