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Antibodies against *Clostridium butyricum* in the children of mothers at risk for gestational diabetes

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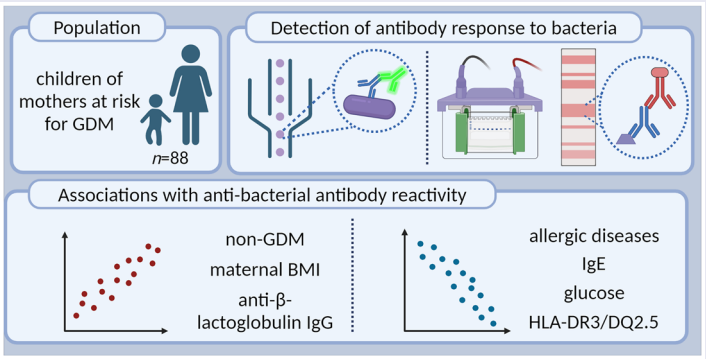
ABSTRACT

Gestational diabetes mellitus (GDM) is linked to an imbalance in gut microbiota composition, which can be transferred to the mother's offspring. *Clostridium butyricum*, known for its health benefits in diabetes and allergy, lacks sufficient data regarding its effect on the immune system's development in the offspring of mothers with GDM. This study assessed antibody responses against *C. butyricum* T2F3 in children of mothers at risk for GDM, involving 88 children aged 1–6 years. Antibody responses were measured with flow cytometry and immunoblot. Lower IgG median fluorescence intensity (MFI) values and fewer IgA and IgG bands against *C. butyricum* were detected in children of mothers with GDM. Maternal body mass index was positively associated with children's IgG MFI and number of IgG bands. Fewer IgA bands were detected in children with higher IgE levels, atopic dermatitis, asthma, and allergic rhinitis. More IgG bands were detected in children with higher anti-β-lactoglobulin IgG levels. Children with autoimmune risk-related HLA-DR3/DQ2.5 had fewer IgA bands, while those with neutral HLA-DR1/DQ5 had higher IgA, but lower IgG MFI. These results indicate that maternal prenatal changes could affect their offspring's immune response against *C. butyricum*. Moreover, *C. butyricum* could have a protective role against allergic sensitization.

GRAPHICAL ABSTRACT



Abbreviations: AU: Arbitrary units; BMI: Body mass index; BSA: Bovine serum albumin; CFU: Colony-forming unit; ELISA: Enzyme-linked immunosorbent assay; FAA: Fastidious Anaerobe

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Agar; FITC: Fluorescein isothiocyanate; GDM: Gestational diabetes mellitus; GWG: Gestational weight gain; HLA: Human leukocyte antigen; I-FABP: Fatty-acid binding protein; kUA/l: Allergen-specific kilo units per liter; MFI: Median fluorescence intensity; OD: Optical density; OGTT: Oral glucose tolerance test; PBS: Phosphate-buffered saline; PVDF: Polyvinylidene fluoride; T1D: Type 1 diabetes; T2D: Type 2 diabetes

1. Introduction

Gestational diabetes mellitus (GDM) is a metabolic disorder which influences the health of both mothers and their children [1,2]. Gestational gut dysbiosis is characteristic of women who develop GDM. Moreover, the imbalance of the maternal microbiota can be transmitted to the offspring, causing abnormalities in the development of their immune system [3–6]. Microbiota dysbiosis at an early age is associated with various immune-mediated diseases and has been shown to promote allergy development [2,3,6,7].

Maternal dysbiosis during GDM has been associated with lower abundance of beneficial gut commensals [7,8]. The vital position of biodiversity in immunoregulatory pathways was described earlier by Nurminen et al. and Roslund et al. who showed that an increase in the skin and gut biodiversity promoted an anti-inflammatory milieu [9,10]. They also found that the gut microflora is impacted by the soil microbiota and the relative abundance of Clostridiales in the gut is affected by a change in biodiversity [9,11].

Early childhood living environment is associated with immune-mediated diseases, such as type 1 diabetes (T1D) and allergic diseases [12–14]. Contact with microbially rich soil containing *Clostridium* sp. promotes immune regulation [11–14]. For example, *C. butyricum* participates in the activation of regulatory T cells, which helps balance type 1 and type 2 T helper cells, thereby preventing pro-allergic reactions and IgE antibody production [15,16]. Moreover, *C. butyricum* is one of the first commensals to colonize the newborn's gut [16]. It is a Gram-positive butyrate producing bacterium that can strengthen the gut barrier, reduce gut permeability, and its metabolites can promote the proliferation of other important gut commensals, such as bifidobacteria and lactobacilli [17,18].

Most studies of *C. butyricum* and immune system-associated diseases, such as allergies, have been conducted by using mouse models [15,19]. However, there is a gap in the knowledge regarding the impact of soil-originating strain, such as *C. butyricum* on humans. Moreover, the presence of *C. butyricum* in the offspring of mothers with GDM and its association with the immune system of these children during the first years of their lives have not been established. The current study aimed to fill in this gap by assessing differences between antibody reactivity towards *C. butyricum* in the children of mothers who developed GDM compared to the children of mothers who did not. Additionally, we aimed to find associations of detected antibody reactivity with the clinical and demographic characteristics of children and mothers, as well as with children's intestinal permeability.

2. Materials and methods

2.1. Study population

The study comprised 88 children, with median age of 1.95 (ranged 1–6 years), born to mothers who were at risk for GDM during their pregnancy according to the Estonian Gynaecologists' Society guidelines and who participated in the GDM study of the University of Tartu during 2014–2019 [20]. The study was approved by the University of Tartu Research Ethics Committee (Estonia; protocols 298/M-21 and 315/M-18) and complied with documents of the Declaration of Helsinki. Written informed consent from the children's parents or guardians was obtained before participating in the study. Among the 88 children included in the study, the mothers of 38 had been diagnosed with GDM and the remaining 50 children acted as the reference. GDM was diagnosed based on the oral glucose tolerance test (OGTT) according to the International Association of Diabetes and Pregnancy Study Groups Consensus Panel [21]. Dietary counseling was provided to all mothers diagnosed with GDM, which was sufficient to maintain their blood glucose levels within the normal range. Venous blood samples were collected from the children at the Women's Clinic of Tartu University Hospital, Estonia (2020–2021), and the separated serum and plasma were stored at −40 and −80 °C, respectively, until further analysis.

2.2. Background characteristics

Background information about the mothers and children was collected from questionnaires. Maternal abnormal gestational weight gain (GWG) was calculated according to the Institute of Medicine and National Research Council Committee guidelines

based on the mother's pre-pregnancy body mass index (BMI) [22]. The GWG values were also considered as abnormal or normal variables. Data about the children's diagnoses of interest [atopic dermatitis (L20) and respiratory-related: allergic rhinitis (J30) and asthma (J45.0 and J45.1) according to ICD-10] were obtained from Electronic Health Records. Neonatal hypoglycemia was diagnosed in accordance with the established monitoring guidelines for Estonia. Because only one child in our study experienced a postnatal infection, we excluded this variable from our analysis due to its low frequency. Blood glucose, C-peptide and vitamin D levels were measured at the United Laboratories of Tartu University Hospital according to their routine protocol. Serum total IgA was detected using enzyme-linked immunosorbent assay (ELISA; Nordic BioSite, Sweden) according to the manufacturer's instructions.

2.3. Intestinal fatty acid-binding protein and anti-β-lactoglobulin antibody detection

To evaluate intestinal epithelial damage, intestinal fatty-acid binding protein (I-FABP) levels were detected from serum samples (diluted 1/10) using the ELISA test kit (HK406-02, Hycult Biotech, the Netherlands). The obtained results were calculated according to the manufacturer's directions and expressed in pg/ml. IgA and IgG antibodies against β-lactoglobulin were measured using ELISA as described by Savilahti et al. with some modifications [23]. Microtiter plates (Nunc PolySorp, Denmark) were coated with bovine lactoglobulin (2 µg/ml; Sigma-Aldrich, USA) in a carbonate-bicarbonate buffer and incubated overnight +4 °C. The plates were washed with phosphate-buffered saline (PBS)-Tween 20 (0.05%) and blocked with 1% normal horse serum in PBS for 1 h at +37 °C. Diluted serum (1/20 in 1% horse serum-PBS) was applied in triplicate (2 coated with and 1 without the antigen) and the plates were incubated for 1 h at +37 °C. After the plates were washed, 100 µl of horseradish peroxidase-conjugated rabbit anti-human IgA (diluted 1/4000; Dako, Glostrup, Denmark) or IgG (diluted 1/4000; Dako) was added and incubated for 1 h at +37 °C. 100 µl of o-phenylenediamine dihydrochloride (Thermo Fisher Scientific, Sweden) in citrate buffer and H_2O_2 (Sigma-Aldrich, USA) was added after washing. The reaction was stopped after 10 min (at room temperature) with 1 N H_2SO_4, and optical density (OD) was measured at 492 nm. Four serum samples positive for IgE antibodies against milk protein (f2, Thermo Fisher Scientific/Phadia, Sweden) were used as a positive reference pool. The detected OD values were used to calculate arbitrary units (AU) using the formula: studied sera (mean OD of two wells – OD without the antigen)/reference pool (mean OD of two wells – OD without antigen).

2.4. IgE sensitization

IgE antibody sensitization to allergens was analyzed with ImmunoCap 100 (Thermo Fisher Scientific/Phadia, Sweden) with the Phadiatop Infant test kit (Thermo Fisher Scientific/Phadia) according to the manufacturer's instructions, with a cut-off value of ≥ 0.35 kUA/L (allergen-specific kilo units per liter) for positivity. In addition, a cut-off of ≥ 0.7 kUA/L was used to identify participants with definite allergic sensitization [24]. The IgE sensitization test included chicken egg, cow's milk, peanut, shrimp, *Dermatophagoides pteronyssinus*, cat, dog, birch, thyme, ragweed and wall pellitory (*Parietaria judaica*) allergens.

2.5. HLA genotyping

PCR-based lanthanide labelled oligonucleotide hybridization with time-resolved fluorometry was used for HLA-DR/DQ genotyping as described previously [25,26]. Based on the data, the DR1/DQ5, DR3/DQ2.5, DR4/DQ8 and DR15/DQ6.2 haplotypes were coded as yes or no variables. The risk for developing autoimmune T1D has been associated with the DR3/DQ2.5 and DR4/DQ8 haplotypes, and protective risk and neutral risk have been regarded as haplotypes DR15/DQ6.2 and DR1/DQ5, respectively.

2.6. Isolation and preparation of C. butyricum

The *C. butyricum* strain T2F3 was isolated from the commercial gardening soil sample no. RG166 T2N5_2019 collected from Finland in spring 2019. The soil "Nurmikkomulta" contained peat and mineral soil, and composted sludge as fertilizer, which was enhanced with composted coniferous bark and composted dung, including chicken dung. The soil samples were cultivated on Fastidious Anaerobe Agar (FAA, Lab M, Heywood, UK) plates at +36.5 °C in an anaerobic glove chamber (5% CO_2, 5% H_2, and 90% N_2; Concept 400, Biotrace, Bridgend, UK) for one week. After isolation, the strain T2F3 was incubated on FAA agar for six hours at the same temperature and anaerobic glove chamber conditions. Depending on the experiment, the *C. butyricum* strain T2F3 was harvested in 20% glycerin solution or PBS at a cell density of 10^8–10^9 CFU/ml and stored at −80 °C until use.

2.7. Detection of C. butyricum surface-bound antibodies by flow cytometry

The method for detecting anti-bacterial antibodies using flow cytometry was adapted from Moor et al. [27] Briefly, *C. butyricum* T2F3 samples stored in a 20% glycerin solution were thawed and washed twice (6000*g*, +4 °C for 10 min) in a sterile filtered 1xPBS solution. The bacterial solution was brought to a concentration of 5×10^6 cells per sample using a PBS-BSA buffer [filtered PBS with 2% bovine serum albumin (BSA, Thermo Fisher Scientific, USA) and 0.02% NaN_3 (Serva, Germany)]. For complement inactivation, thawed serum samples were heated at +56 °C for 30 min and centrifuged at 16,000*g* at +4 °C for 5 min. The supernatant was passed through a 0.22 µm spin filter column (VWR Centrifugal Filters, US) and diluted with a PBS-BSA buffer to serial dilutions of 1/81, 1/243, 1/729 and 1/2187. The diluted serum samples (25 µl) were incubated with 25 µl of bacteria for 15 min at room temperature and washed twice with PBS-BSA (3320*g*, +4 °C for 10 min). Next, 50 µl of 1/1 pooled fluorophore-conjugated goat anti-human IgA [diluted 1/200; fluorescein isothiocyanate (FITC) conjugated, Jackson ImmunoResearch, USA] and IgG (diluted 1/214; Alexa Fluor 647 conjugated, Jackson ImmunoResearch, USA) antibodies were added and incubated for 15 min. The washing step was repeated twice. To visualize bacteria, 1/1000 diluted SYTOX™ orange nucleic acid stain (Invitrogen™) was added together with 1/1000 diluted Triton X-100 (Sigma-Aldrich, USA) and incubated for 20 min. All incubation steps were performed in the dark at room temperature. The samples were acquired using LSRFortessa™ (BD Biosciences, USA) with 10,000 target events recorded for each sample. Flow cytometry data was analyzed using the FACSDiva™ version 6.2 (BD Biosciences, USA). For each sample, median fluorescence intensity (MFI) for IgA and IgG was recorded, the obtained values reflecting antibody amount against *C. butyricum*'s surface epitopes. To ensure that all MFI values from the analysis were positive, the scale's zero point was shifted to the lowest MFI value and all MFI values were subsequently adjusted. In addition, the proportions of bacteria bound with only IgA antibodies, with only IgG antibodies, or with both IgA and IgG antibodies per 10,000 bacteria were recorded.

2.8. Detection of C. butyricum specific antibodies by immunoblot assay

For the immunoblot experiment, the bacterial cells stored in PBS were washed three times with the PBS buffer and disrupted with 0.1 mm glass beads (30 sec of cell disruption, 10 min on ice and repeated approximately 10 times; Biospec Products, USA). The protein assay solution (Bio-Rad, USA) was used to detect protein concentration in samples, and 100 µg protein was loaded on a gradient gel with the SDS-PAGE sample buffer [62.5 mM Tris (pH 6.8), 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerin, a few grains of bromophenol blue]. Antigens were separated for the immunoblot according to Nilsson et al. using the vertical electrophoresis system SE-600 (Hoefer, San Fransisco, CA, USA) and transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 µm pore size) using a semi-dry electroblotter (Hoefer) [28]. All blue 10–250 kDa molecular weight markers (Bio-Rad, USA) were used. Plasma was diluted in the incubating buffer [1.25 g/L gelatin hydrolysate, 0.25 g/L Tween-20, 6.1 g/L NaCl (Merck, USA), 0.06 g/L Tris Base] 1/50 for IgA and 1/100 for IgG detection, and incubated overnight on a shaker at +4 °C. Horseradish peroxidase-conjugated rabbit polyclonal anti-human IgA or IgG antibodies (Invitrogen, USA) were applied at 1/2000 dilution and incubated for two hours at constant shaking at room temperature. The reaction was stopped using distilled water, and the number of bands on the PVDF membrane was counted. The higher the band count, the more IgA or IgG antibody reactions were found in the sample.

2.9. Statistical analysis

Categorical characteristics were compared using the χ^2 test or Fisher's exact test, and continuous characteristics were compared using the Welch two-sample *t*-test for a normal distribution and the Wilcoxon rank sum test for a non-normal distribution. Correlations between children's and mother's clinical data and bacterial antibody response values were found using Spearman's correlation. Linear regression with antibody parameters on the $\log_2$ scale was employed to ensure a better linear fit. In addition, Poisson regression was used. Regression models were adjusted for the child's age and sex, if not stated otherwise. p-value < 0.05 was considered statistically significant. Statistical analysis was done using R (version 4.3.1, The R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Children's clinical characteristics and antibody parameters

The background characteristics were similar for children born to mothers diagnosed with GDM and for children born to the mothers without the diagnosis, except for a few differences (Table 1). The HLA-DR3/

DQ2.5 haplotype was significantly more common in children of the non-GDM group ($p=0.026$). Additionally, in the GDM group children had higher levels of anti-β-lactoglobulin IgA antibodies ($p=0.015$). Although the IgE test results at ≥ 0.35 kUA/L were similar between the compared groups, there was a trend for a higher proportion of children with positive IgE at ≥ 0.7 kUA/L in the GDM group ($p=0.053$). As expected, maternal OGTT values ($p<0.001$), as well as pre-pregnancy and antepartum BMI ($p=0.014$ and $p=0.035$, respectively) were higher in the GDM group.

Comparison of the data about children's antibody reaction between children born to the mothers with GDM and those without GDM is shown in ESI Table S1. The proportion of bacteria with antibodies bound to the bacterial surface is shown at the serum dilution of 1/243, which best distinguished both IgA and IgG antibody responses. Serum dilution of 1/2187 was excluded from statistical analysis for it being too dilute. Overall, the antibody reactivity detected with flow cytometry was similar between the two groups. At the same time, bacteria bound with both IgA and IgG antibodies were detectable in only a few children (median values for both groups were 0%; Table S1). Immunoblot analysis revealed a trend for higher number of IgA bands in children belonging to the non-GDM group (median 12 *vs* 16, $p=0.056$; Table S1).

3.2. Correlations

Figure 1 shows Spearman's correlation between antibody values and children's clinical characteristics. With children's increasing age, percentage of bacteria bound with IgA ($r=0.22$, $p=0.044$) and values of bacteria-binding IgA and IgG MFI (at dilution 1/81, $r=0.21$, $p=0.049$ and $r=0.38$, $p<0.001$, respectively) increased. On the other hand, number of IgA and IgG bands correlated negatively with age ($r=-0.53$, $p<0.001$ and $r=-0.67$, $p<0.001$, respectively). Vitamin D levels correlated with IgA and IgG MFI values (at dilution 1/81, $r=-0.22$, $p=0.044$ and $r=-0.25$, $p=0.019$, respectively). Total IgA levels had inverse correlation with number of IgA and IgG bands ($r=-0.35$, $p=0.002$ and $r=-0.32$, $p=0.004$, respectively), but positive correlation with IgG MFI values (at dilution 1/81, $r=0.22$, $p=0.043$). Additionally, significant correlation was found between anti-β-lactoglobulin IgG antibodies and number of IgG bands ($r=0.31$, $p=0.005$).

3.3. Regression models

In regression analysis, only the data from the serum dilution 1/243 was used, as it best distinguished both IgA and IgG antibody responses. Crude models (data not shown) showed that living in urban or rural areas did not affect children's antibody reactivity against *C. butyricum* T2F3. However, children's daycare attendance was associated with higher IgG MFI values ($\beta=0.77$, $p=0.003$), but with lower number of IgA and IgG bands ($\beta=-0.23$, $p<0.001$ and $\beta=-0.53$, $p<0.001$, respectively), compared to children staying at home. Additionally, inverse associations with number of IgA and IgG bands were found for children born *via* scheduled cesarean section compared to emergency cesarean section ($\beta=-0.36$, $p=0.008$ and $\beta=-0.48$, $p=0.002$, respectively). Also, more IgA and IgG bands were detected in children who were breastfed during the study period ($\beta=0.31$, $p<0.001$ and $\beta=0.36$, $p<0.001$, respectively). I-FABP values were positively associated with number of IgA bands ($\beta=0.0002$, $p=0.010$).

In adjusted models, we found that after adjustment for pre-pregnancy BMI, in addition to child's age and sex, the children of mothers with GDM showed lower IgG MFI values ($\beta=-0.52$, $p=0.028$) and fewer IgA and IgG bands ($\beta=-0.26$, $p<0.001$ and $\beta=-0.30$, $p<0.001$, respectively). Similarly, higher maternal pregnancy glucose levels after 1h of the OGTT were associated with fewer number of IgG bands in their children (model adjusted for same covariates, $\beta=-0.04$, $p=0.049$). On the other hand, in a model adjusted for maternal GDM diagnosis and child's age and sex, the children of mothers with higher pre-pregnancy BMI showed a higher IgG MFI value ($\beta=0.05$, $p=0.025$) and more IgG bands ($\beta=0.02$, $p=0.002$). More specifically, the children of obese mothers had more IgG bands compared to the children of mothers with normal weight ($\beta=0.22$, $p=0.012$). However, children whose mothers gained more weight during gestation had fewer IgA and IgG bands ($\beta=-0.01$, $p=0.020$ and $\beta=-0.02$, $p=0.003$, respectively).

Additionally, adjusted models revealed fewer IgA bands in children diagnosed with either atopic dermatitis ($\beta=-0.22$, $p=0.003$) or respiratory-related diagnoses ($\beta=-0.23$, $p=0.032$). An inverse association was also found between number of detected IgA bands and the IgE levels ($\beta=-0.06$, $p=0.029$), as well as between number of detected IgA bands and the level of total IgA ($\beta=-0.09$, $p=0.005$). Children who had hypoglycemia after birth had lower percentages of bacteria bound with IgA ($\beta=-1.13$, $p=0.033$), in a model adjusted for maternal GDM diagnosis and child's age and sex. Children with the DR1/DQ5 HLA haplotype showed higher values of IgA but lower IgG MFI ($\beta=0.52$, $p=0.019$ and $\beta=-0.58$, $p=0.033$, respectively), in a model adjusted for maternal GDM diagnosis and child's age and sex.

Table 1. Comparison of the clinical data of children and their mothers in the GDM and non-GDM groups.

Clinical characteristics	GDM group ($n=38$)	Non-GDM group ($n=50$)	p-value
Children			
Age (years)	2.3 (1–5.8)	1.9 (1–6.3)	0.686
Sex (male)	22 (57.9%)	26 (52%)	0.738
Mode of birth			
Vaginal	31 (81.6%)	39 (78%)	0.584
Scheduled cesarean section	2 (5.3%)	6 (12%)	
Emergency cesarean section	5 (13.2%)	5 (10%)	
Hypoglycemia	9 (23.7%)	8 (16%)	0.528
Residence			
Urban area	17 (44.7%)	29 (58%)	0.309
Rural area	21 (55.3%)	21 (42%)	
Daycare attendance	19 (50%)	22 (44%)	0.798
Breast milk consumption	7 (18.4%)	15 (30%)	0.320
Duration of breastfeeding (months)	10 (5–16.3)	13 (11–17)	0.088
Laboratory analysis			
Total IgA (mg/mL)	1.2 (1–1.4)	1.3 (0.9–2.2)	0.494
Glucose (mmol/L)	4.5 (4.2–5)	4.6 (4.2–4.9)	0.858
C-peptide (nmol/L)	0.62 (0.38–0.99)	0.60 (0.38–0.81)	0.139
Vitamin D (nmol/L)	73.4 (58.3–94.6)	65.3 (56.1–77.9)	0.086
I-FABP (pg/ml)	427 (293.8–712.8)	522.8 (298.2–872.2)	0.667
Anti-β-lactoglobulin IgA (AU)	0.25 (0.14–0.56)	0.12 (0.05–0.27)	**0.015**
Anti-β-lactoglobulin IgG (AU)	0.72 (0.51–0.9)	0.59 (0.38–0.9)	0.207
IgE sensitization			
IgE (kUA/L)	0.13 (0.05–0.56)	0.12 (0.05–0.29)	0.435
IgE test ≥ 0.35 kUA/L	13 (34.2%)	11 (22%)	0.348
IgE ≥ 0.7 kUA/L	8 (21.1%)	3 (6%)	0.053
Diagnosis of interest			
Atopic dermatitis	12 (31.6%)	10 (20%)	0.320
Respiratory-related	4 (10.5%)	8 (16%)	0.542
HLA haplotype			
DR1/DQ5	8 (21.1%)	11 (22%)	1
DR3/DQ2.5	3 (7.9%)	13 (26%)	**0.026**
DR4/DQ8	4 (10.5%)	6 (12%)	1
DR15/DQ6.2	12 (31.6%)	14 (28%)	1
Mothers during pregnancy			
Age (years)	31.6 (21–40)	32 (19–44)	0.608
Oral glucose tolerance test (mmol/L)			
Fasting glucose	5 (4.6–5.3)	4.6 (4.4–4.9)	**< 0.001**
1h glucose	10.1 (8.6–10.5)	7.1 (5.9–7.9)	**< 0.001**
2h glucose	7.5 (6.6–8.5)	5.8 (4.9–6.5)	**< 0.001**
Body mass index (BMI; kg/m^2)			
Pre-pregnancy	26.1 (23.7–29.6)	23.6 (21.2–27.9)	**0.014**
Antepartum	29.7 (27.6–36.1)	29 (26–31.7)	**0.035**
Pre-pregnancy BMI factor (kg/m^2)			
Underweight (BMI < 18.5)	1 (2.6%)	2 (4%)	0.354
Normal weight (BMI 18.5–24.9)	15 (39.5%)	28 (56%)	
Overweight (BMI 25–29.9)	13 (34.2%)	12 (24%)	
Obese (BMI ≥ 30)	9 (23.7%)	7 (14%)	
Gestational weight gain (kg)	11 (8–15)	13.1 (10.2–17)	0.357
Abnormal gestational weight gain	20 (52.6%)	28 (56%)	0.862

Statistically significant differences ($p < 0.05$) are highlighted in bold.

Additionally, children with the DR3/DQ2.5 haplotype had fewer IgA bands (model adjusted for the same covariates, $\beta = -0.22$, $p=0.011$). Furthermore, the child's vitamin D and random blood glucose levels were inversely associated with IgG MFI values ($\beta = -0.007$, $p=0.041$) and with number of detected IgG bands ($\beta = -0.17$, $p=0.007$), respectively. However, there occurred positive association with children's anti-β-lactoglobulin IgG antibody levels and IgG MFI values and number of IgG bands ($\beta=0.76$, $p=0.049$ and $\beta=0.37$, $p=0.001$, respectively).

4. Discussion

GDM has been shown to be associated with microbiota dysbiosis [2,7]. When transferred to the mother's offspring, these changes in the gut can cause abnormalities in the development of the immune system with several consequences, including allergy development in these children [3–6]. However, the knowledge of the effect of *C. butyricum* on the immune system in the offspring of GDM mothers is inadequate. This study assessed associations between antibody reactions against soil-isolated *C. butyricum* T2F3 in children born to mothers at risk for GDM during pregnancy. In addition, we aimed to find associations of *C. butyricum*'s antibody reactivity with children's and mothers' background characteristics and children's intestinal permeability.

We found that children of mothers with GDM showed lower IgG MFI values and fewer IgA and IgG bands reacting with *C. butyricum,* which suggests that children in the GDM group may have a diminished anti-*C. butyricum* antibody response.

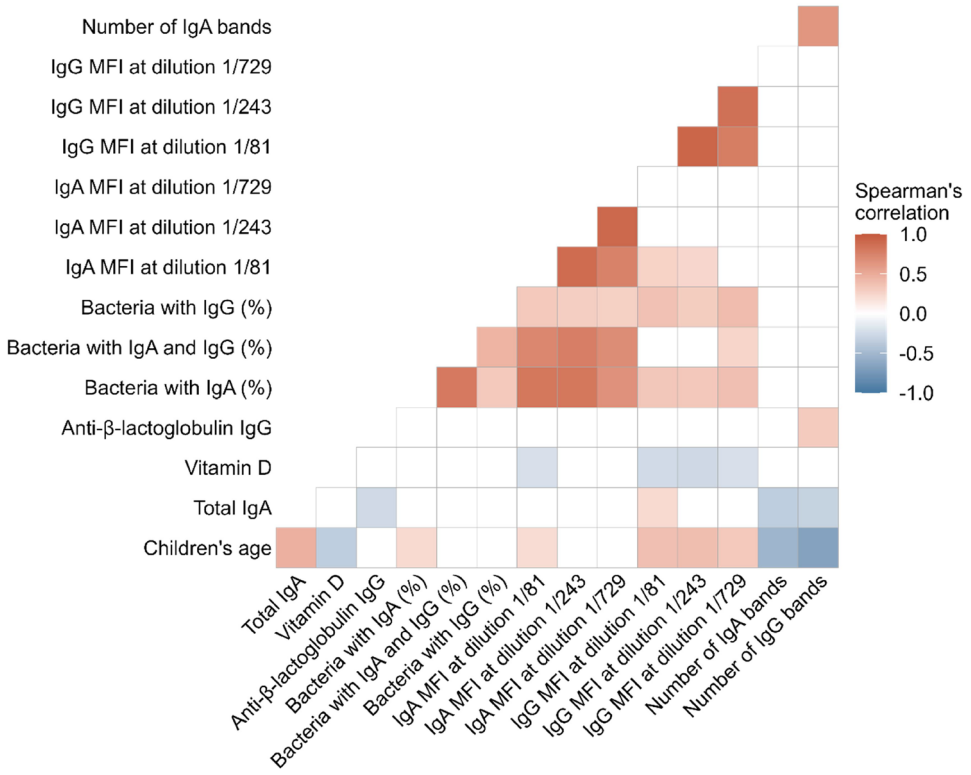

Figure 1. Spearman's rank correlation between children's clinical data and parameters of *C. butyricum*'s antibody reactivity. Bacteria with antibodies (%) represent the proportion of bacteria with either surface-bound IgA, IgG, or both IgA and IgG (at dilution 1/243). MFI indicates the intensity of antibody fluorescence against bacterial surface proteins. The parameters obtained from immunoblot experiment are presented as the number of IgA and IgG bands. Only the data indicating significant correlations with parameters of antibody reactivity are shown. Red color indicates positive correlation and blue color indicates negative correlations ($p < 0.05$). MFI, median fluorescence intensity.

Paun et al. demonstrated that a lower antibody response directed towards gut commensals was associated with their lower abundance in the gut and vice versa [29]. This association is likely driven by the ability of commensal gut microbes to induce serum IgA and IgG responses, which in turn facilitate colonization and functioning [27]. In women with GDM, reduced presence of species annotated to *Clostridium* (*sensu stricto*) has been reported [7,8]. However, to our knowledge, no studies have previously reported *C. butyricum*'s abundance in the offspring of these women. Therefore, we do not know whether the vertical transmission of *Clostridium* species from non-GDM mothers to their offspring is greater than it is from mothers with GDM, which could then cause increased exposure to *Clostridium* and hence stronger immune response. This hypothesis needs to be confirmed by future studies.

Interestingly, both maternal and child's blood glucose levels were inversely associated with number of detected IgG bands reacting with *C. butyricum*. Furthermore, children with postnatal hypoglycemia—potentially influenced by maternal hyperglycemia—had lower percentage of bacteria coated with IgA, suggesting a potential link between glucose regulation and immune interactions with *C. butyricum*. Several studies have described the link between *C. butyricum* and diabetes. An anti-diabetic effect of *C. butyricum* CGMCC0313.1 was reported in a type 2 diabetic (T2D) mouse model where *C. butyricum* reduced glucose levels and improved insulin resistance [30]. In addition, Zhou et al. reported decreased abundance of *C. butyricum* in the gut of diabetic mice [31]. In humans, negative association was found between *Clostridium* cluster IV and 2h plasma glucose levels in women with GDM, as well as between certain *Clostridium* species and fasting glucose levels in subjects with T2D [7,32]. Therefore, *C. butyricum* could protect against diabetes development by reducing blood glucose levels in persons at risk for diabetes, including the offspring of mothers with GDM.

In addition to hyperglycemia, several GDM risk factors are also associated with changes in the mother's and child's gut microbiota. Su et al. showed that maternal higher pre-pregnancy and antepartum BMI were associated with higher abundance of the *Clostridium* genus in the newborn's gut [33]. Therefore, we aimed to investigate whether maternal BMI and GWG could also contribute to children's antibody reactivity against *C. butyricum*. We found that the children of mothers with higher pre-pregnancy

BMI had higher *C. butyricum* IgG MFI and higher number of detected IgG bands. Furthermore, maternal pre-pregnancy obesity was associated with an increased number of IgG bands. These associations might indicate increased exposure to this bacterium in these children. Notably, higher maternal GWG was associated with a reduced number of detected IgA and IgG bands reacting with *C. butyricum*. However, considering that women with lower pre-pregnancy BMI typically experience greater weight gain during pregnancy compared to those with higher BMI [34], our findings align with the above observation of a positive correlation between maternal pre-pregnancy BMI and increased IgG reactivity.

Another important finding was that we detected fewer IgA bands in children diagnosed with either atopic dermatitis or respiratory-related diagnoses. Decreased seroreactivity against commensals is observed in allergy-prone children [29,35]. Therefore, lower IgA in children with atopic dermatitis might be linked to reduced *Clostridium* abundance, as previously reported [36]. This may also reflect diminished mucosal immune stimulation and barrier dysfunction, consistent with Dzidic et al.'s finding of weaker IgA responses to gut commensals in allergic children, indicating impaired mucosal barrier function [37]. Also, *C. butyricum* has demonstrated allergy-preventive potential [19,38,39], modulating regulatory T cells *via* IL-10 production [16,19,39] which is further supported by our observation of a negative association between the number of *C. butyricum*-reactive IgA bands and IgE level. Increased intestinal permeability can also be indicated by increased levels of IgA and IgG antibodies to the food allergen β-lactoglobulin [40,41]. While children of mothers with GDM exhibited higher anti-β-lactoglobulin IgA levels, we surprisingly found no corresponding increase in I-FABP, a marker of intestinal barrier dysfunction [42]. Similarly, despite *C. butyricum's* known gut-strenghtening properties [16], I-FABP levels were not associated with anti-*C. butyricum's* antibody responses. Only increased anti-β-lactoglobulin IgG antibody levels were accompanied by higher anti-*C. butyricum's* IgG MFI values and number of IgG bands. However, further research, including the assessment of other intestinal permeability markers like zonulin [41], is needed to clarify the interplay between β-lactoglobulin sensitization, *C. butyricum*, and intestinal barrier function.

Previous research has demonstrated that autoimmune-associated HLA haplotypes influence the colonization of gut bacteria and play a crucial role in regulating the immune response to these microbes [29,43,44]. Consistent with our findings, which revealed a decrease in *C. butyricum*-specific IgA bands among children carrying the HLA-DR3/DQ2.5 haplotype, Paun et al. also noted diminished anti-commensal antibody responses in individuals with T1D who carried either the DR3 or DR4 haplotype [29]. These haplotypes have previously been linked to the decreased abundance of specific commensal bacteria, such as bifidobacteria. Furthermore, Berryman et al. reported a dose-dependent relationship, where the relative abundance of *Bifidobacterium* was the lowest in individuals homozygous for the DR4/DQ8 haplotype, but the highest in those homozygous for the haplotype DR1/DQ5 [43]. They suggested that HLA variants associated with increased risk for autoimmune diseases may act as gatekeepers in the gut, influencing the balance of beneficial microbes and contributing to dysbiosis [43]. Our observation of higher IgA MFI values in children with the HLA-DR1/DQ5 haplotype, further elaborates these findings. On the other hand, Vitamin D is known to modulate the adaptive and innate response of the immune system [45]. Some studies have shown that as vitamin D can also affect anti-microbial response, it could suppress the immunoglobulin production suppressing these bacteria [46,47]. Our results are consistent with this hypothesis, as we detected higher vitamin D levels in children with lower IgG MFI against *C. butyricum*.

Since *C. butyricum* T2F3 was isolated from gardening soil we expected that children living in urban areas would be more exposed to the studied strain. However, there were no differences in antibody reactivity towards *C. butyricum* between children living in rural and urban areas, which might indicate that children in the compared groups were equally exposed to the bacterium.

To assess children's antibody response to *C. butyricum*, we used flow cytometry and immunoblot assays, revealing somewhat divergent results. We found a negative correlation between children's age and the number of IgA and IgG bands detected *via* immunoblot but a positive correlation between age and IgA and IgG MFI values measured by flow cytometry. This difference may indicate that antibodies targeting conformational epitopes on intact bacterial cell surfaces, detected *via* flow cytometry, become more prevalent with age. On the other hand, immunoblot assay uses bacterial cell homogenate and allows the detection of antibodies bound to lysed bacterial components, rather than intact cell surfaces [28]. Since *C. butyricum* can exist as spores in the gut *in vivo* [16,48], potentially altering exposed epitopes, we ensured that the flow cytometry protocol was completed within a short time frame (~ 3 h) before acquiring samples. This precaution helped prevent *C. butyricum* from reaching a significant

level of spore formation. Consequently, the obtained results only describe immune reactivity against the vegetative form of *C. butyricum*.

The use of two different methods for analyzing antibody reactivity against *C. butyricum* can be seen as a strength of the study, enabling to detect both bacterial surface-specific and intracellular epitopes. Furthermore, the children of the study groups belonged to various age groups, facilitating assessment of immunological changes against *C. butyricum* across different age ranges. However, the small sample size poses a limitation the study. With a limited number of participants, we were only able to categorize children based on the presence of the diagnoses of interest. Categorizing them according to symptoms would have likely led to an underpowered evaluation. Larger sample sizes in future studies should be used to explore immune responses based on allergic symptoms. Additionally, there were no samples from children younger than one year, which would have enabled us to better study the maternal effect on the offspring's microbiota and immune systems. Furthermore, we did not account for maternal nutrition during pregnancy, a factor known to significantly impact the offspring's microbiota and immune system. These limitations may have restricted our ability to fully assess maternal contributions to these developmental processes.

5. Conclusions

The children of mothers who were diagnosed with GDM had lower IgG MFI and fewer IgA and IgG bands against *C. butyricum*. These results indicate that maternal health during pregnancy influences the child, subsequently affecting the response of offspring's immune system to *C. butyricum*. Factors such as maternal glucose levels, BMI and child's HLA haplotypes, glucose, vitamin D and anti-β-lactoglobulin IgG antibody levels were also found to affect antibody reactivity. In addition, the results suggest a possible protective role of *C. butyricum* against developing atopic dermatitis and respiratory-related diagnoses in children. Together, these findings highlight the complex interplay between maternal health, microbial exposure and offspring immunity and underline the importance of further research to understand and potentially mitigate the risk of immune-related conditions in children born to mothers at risk for GDM.

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Authors' contributions

C.P.: Writing – original draft, formal analysis, visualization. C.P., A.T., K.A., A.B., T.V., I.T. and H.J.: Investigation. A.K. and A.B.: Participant recruitment. A.S., M.I.R. and S.K.: Resources. R.U. and A.T.: Project administration, supervision, writing – review and editing. R.U.: Funding acquisition.

Disclosure statement

There are no conflicts to declare.

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Data availability

Immunoblot data for this article are available at DataDOI at [https://doi.org/10.23673/re-470]. Personal data collected from human participants is not publicly available due to confidentiality restrictions.

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