Diabetologia (2023) 66:241–246
https://doi.org/10.1007/s00125-022-05805-3

SHORT COMMUNICATION

Changes in the gut bacteriome upon gluten-free diet intervention do not mediate beta cell preservation

Vit Neuman[1] · Stepanka Pruhova[1] · Michal Kulich[2] · Stanislava Kolouskova[1] · Jan Vosahlo[3] · Martina Romanova[3] · Lenka Petruzelkova[1] · Jaroslav Havlik[4] · Anna Mascellani[4] · Svatopluk Henke[5] · Zdenek Sumnik[1] · Ondrej Cinek[1]

Received: 23 April 2022 / Accepted: 19 August 2022 / Published online: 4 October 2022

Abstract

Aims/hypothesis We previously detected indications that beta cell function is protected by gluten-free diet (GFD) introduced shortly after the onset of childhood type 1 diabetes. The present aim was to assess whether GFD was associated with changes in the gut bacteriome composition and in its functional capacity, and whether such changes mediated the observed effects of GFD on beta cell function.

Methods Forty-five children (aged 10.2 ± 3.3 years) were recruited into a self-selected intervention trial primarily focused on determining the role of GFD on beta cell preservation (ClinicalTrials.gov NCT02867436). Stool samples were collected prior to the dietary intervention and then at 3-month intervals. A total of 128 samples from the GFD group and 112 from the control group were analysed for bacteriome 16S rDNA community profiles, the bacteriome functional capacity was predicted using PICRUSt2 and actual gut metabolome profiles measured using NMR. Intestinal permeability was assessed using serum zonulin concentrations at 1, 6 and 12 months and lactulose/mannitol tests at the end of intervention. Dietary questionnaires were used to ensure that the dietary intervention did not result in differences in energy or nutrient intake.

Results The bacteriome community composition changed during the intervention with GFD: of abundant genera, a 3.3-fold decrease was noted for *Bifidobacterium* genus (adjusted $p=1.4 \times 10^{-4}$ in a DESeq2 model, $p=0.026$ in generalised estimating equations model), whereas a 2.4-fold increase was observed in *Roseburia* (adjusted $p=0.02$ in DESeq2 model, $p=0.002$ in generalised estimating equations model). The within-sample (alpha) diversity did not change, and there was no statistically significant clustering of GFD samples in the ordination graphs of beta diversity. Neither of the genera changes upon GFD intervention showed any association with the pace of beta cell loss ($p>0.50$), but of the remaining taxa, several genera of Bacteroidaceae family yielded suggestive signals. The faecal metabolome profile ordination correlated with that of bacteriomes but did not associate with GFD or categories of beta cell preservation. There was no indication of changes in gut permeability.

Conclusions/interpretation The bacteriome reacted to GFD, but the changes were unrelated to the pace of beta cell capacity loss. The previously observed moderately protective effect of GFD is therefore mediated through other pathways.

Keywords Gluten-free diet · Gut bacteriome composition · Gut metabolome · Intestinal permeability · Paediatric type 1 diabetes

✉ Vit Neuman
Vit.Neuman@fnmotol.cz

[1] Department of Pediatrics, 2nd Faculty of Medicine, Charles University and Motol University Hospital, Prague, Czechia

[2] Department of Probability and Mathematical Statistics, Faculty of Mathematics and Physics, Charles University, Prague, Czechia

[3] Department of Pediatrics, 3rd Faculty of Medicine, Charles University and Kralovske Vinohrady University Hospital, Prague, Czechia

[4] Department of Food Science, Czech University of Life Sciences, Prague, Czechia

[5] Department of Carbohydrates and Cereals, University of Chemistry and Technology, Prague, Czechia

Research in context

What is already known about this subject?

- Gluten-free diet (GFD) might be associated with slower pace of beta cell function decline in children with newly diagnosed type 1 diabetes
- GFD can change the gut microbiota in both healthy adults and adults with coeliac disease

What is the key question?

- Can GFD change the gut microbiome, its metabolic capacity or intestinal permeability in children with newly diagnosed type 1 diabetes and are these changes associated with the pace of beta cell function decline?

What are the new findings?

- The abundance of two genera changed with the introduction of GFD; while the *Bifidobacterium* genus decreased, the *Roseburia* genus increased its relative abundance
- Neither these nor any other genera were associated with the pace of beta cell decrease
- The introduction of GFD did not lead to changes in the gut metabolome or the intestinal permeability

How might this impact on clinical practice in the foreseeable future?

- Despite the ability of GFD to change the gut microbiome, it is unlikely that its association with slower pace of beta cell function decline is mediated through these changes

Abbreviations

GFD Gluten-free diet
GIP Gluten immunogenic peptides
L/M Lactulose to mannitol

Introduction

In our previous work, we observed a borderline yet detectable effect of gluten-free diet (GFD) on residual beta cell capacity, metabolic control and the length of the partial remission period in children shortly after type 1 diabetes onset [1]. It is presently not known whether such effects might be mediated through the composition or function of the human gut microbiome (which changes upon introduction of this diet [2]), or through the gut permeability (affected by both gluten [3] and microbiota [4]).

The aim of the present study was to investigate samples and data obtained in our previous interventional trial [1] and to test whether GFD is associated with changes in gut bacteriome composition, in its function and in intestinal permeability, and whether such changes mediate the previously observed effects of GFD on the pace of beta cell loss.

Methods

Forty-five children (aged 10.2 ± 3.3 years) were recruited into a self-selected intervention trial primarily focused on determining the role of GFD on beta cell preservation (ClinicalTrials.gov NCT02867436). The detailed study population characteristics have been described previously [1]. This was a non-randomised dietary interventional trial using GFD in children recently diagnosed with type 1 diabetes. The study was completed per protocol by 22 GFD-compliant participants and 19 control participants (electronic supplementary material [ESM] Methods 1.1 and ESM Table 1). The primary study outcome was the AUC of the C-peptide levels in the standard mixed-meal tolerance test (MMTT) [5], assessed at 6 and 12 months and expressed as the percentual decline relative to the baseline test. In an adjusted longitudinal analysis of all three time points, the C-peptide AUC declined more slowly in the GFD group than in the control participants.

To explore the faecal bacteriome as a mediator of the above-mentioned effect of the GFD, we first: (1) explored associations of GFD with changes in the faecal bacteriome community composition and with the faecal metabolome; then (2) tested whether the observed changes are associated with the pace of beta cell loss (the hypothetical taxa or metabolites being a vertex of the mediator effect analysis in ESM Fig. 1a); and then (3) if such outcome-associated taxa or metabolites had been found, formal statistical testing was planned of the mediated (i.e. indirect, intermediate) effect of the bacteriome on the pathway between GFD and beta cell loss (ESM Fig. 1b). The collection of samples, extraction of DNA, PCR amplification of the V4 region of the 16S rDNA gene, amplicon sequencing, bioinformatic and statistical

analyses were performed according to published protocols [6, 7], and are detailed in ESM Methods 1.2. The stool sample metabolome was explored by NMR spectroscopy: ^{1}H NMR spectra were recorded and processed as previously described [8] and detailed in ESM Methods 1.3.

The intestinal permeability was assessed by two different methods: (1) urine lactulose to mannitol (L/M) excretion ratio was measured at the 12-month visit as described by Sequeira et al [9], using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) on a CarboPac MA1 column (Dionex, USA); and (2) as a presumed marker of human intestinal permeability [10], the human protein zonulin was tested in serum samples collected at visits at 1, 6 and 12 months using Human Zonulin ELISA kit (PromoCell, Heidelberg, Germany).

Nutrient intake and intervention adherence were assessed using 3-day nutritional questionnaires collected at each visit. The daily nutrient and energy intake were assessed by NutriServis PROFI software (Forsapi, Prague, Czechia), and compared between intervention and control participants using two-sample Welch *t* tests at 6 and 12 months to ensure that the GFD intervention was not accompanied by other, unwanted changes in the diet. Full dietary data were available for 32 compliant participants (15 GFD group, 17 control group) at 6 months and 30 (13 GFD group, 17 control group) at 12 months.

Every participant was tested for protocol adherence at three or more time points: the presence of faecal gluten immunogenic peptides (GIP) was assessed using the iVylisa GIP-S kit (Biomedal, Sevilla, Spain) [11]. Participants were not specifically reminded about this testing, although it had been covered in the written informed consent. The testing was performed retrospectively, and GIP positivity in any sample taken while on the GFD was considered non-compliance and resulted in exclusion of the child from the statistical analysis.

The study followed the STROBE guidelines [12] and was approved by the institutional Ethics Committee. The study protocol was registered at ClinicalTrials.gov (NCT 02867436). Written informed consent was granted by all participants' parents/caregivers. The parents/caregivers of the participants in the intervention group were financially reimbursed for the added costs of the GFD.

Results

Participants and their samples for bacteriome and metabolome Of the 45 children participating in the study, four in the GFD intervention group were positive for GIP in stool, and were consequently excluded from the per-protocol analysis owing to non-adherence. In the control group, detectable amounts of GIP were present in all of the tested stool samples. The remaining 41 study participants were compliant with the intervention protocol (22 from the GFD group, 19 from the control group; detailed in ESM Results 2.1). Here we report analyses of 240 stool samples collected throughout the study.

GFD intervention and the gut bacteriome The alpha (within-sample) diversity of bacteriomes did not appreciably change upon applying GFD (ESM Fig. 2). Analyses of the overall bacteriome community patterns did not reveal appreciable changes upon GFD intervention, as assessed by transformation-based redundancy analysis, non-metric distance-based ordination or permutational multivariate ANOVA (ESM Results 2.2).

Individual taxonomic components of the bacteriome were tested in two tiers: first, we used sensitive DESeq2 modelling, then candidate taxa identified therein were confirmed in models for longitudinal data (details in ESM Results 2.3). *Bifidobacterium* genus decreased 3.3-fold while on GFD (Fig. 1a,b; adjusted $p=1.4 \times 10^{-4}$ in a DESeq2 model, $p=0.026$ in generalised estimating equations; this association projected upwards to phylum Actinobacteria), whereas *Roseburia* increased 2.4-fold on GFD (Fig. 1c,d; adjusted $p=0.02$ in DESeq2 model, $p=0.002$ in generalised estimating equations).

The gut bacteriome and beta cell residual function If the previously observed effects of GFD on beta cell preservation were mediated by the bacteriome, then not only would the bacteriomes change upon the introduction of GFD, but these bacteriome changes would also be associated with the preservation of beta cell function. This was not the case: neither *Bifidobacterium* ($p=0.86$) nor *Roseburia* ($p=0.83$) were associated with the pace of decline of the beta cell function. Nor was there any convincing association with the residual beta cell function among the remaining taxa: nominally significant association signals were noted for the Bacteroidaceae family (nominal uncorrected $p=0.013$) whose positive association with better beta cell function was likely due to such associations of the genus *Bacteroides* ($p=0.013$, with the strongest contribution from amplicon sequence variants belonging to *B. faecis* or *B. thetaiotaomicron*). Further suggestive direct association was noted for *Odoribacter* ($p=0.011$) and its family Marinifilaceae ($p=0.0063$). The quantity of these taxa is plotted against the percentual preservation of beta cell function in Fig. 2a,b. The beta cell preservation was not associated with alpha diversity of the bacteriome (ESM Fig. 3) nor with overall bacteriome community patterns (ESM Fig. 4 and ESM Results 2.4). The above-mentioned body of evidence together indicates that the composition of the bacteriome did not act as an intermediate in the previously detected link between GFD and beta cell preservation.

The gut metabolome The NMR gut metabolome analysis revealed the presence of 31 compounds, whereas some of

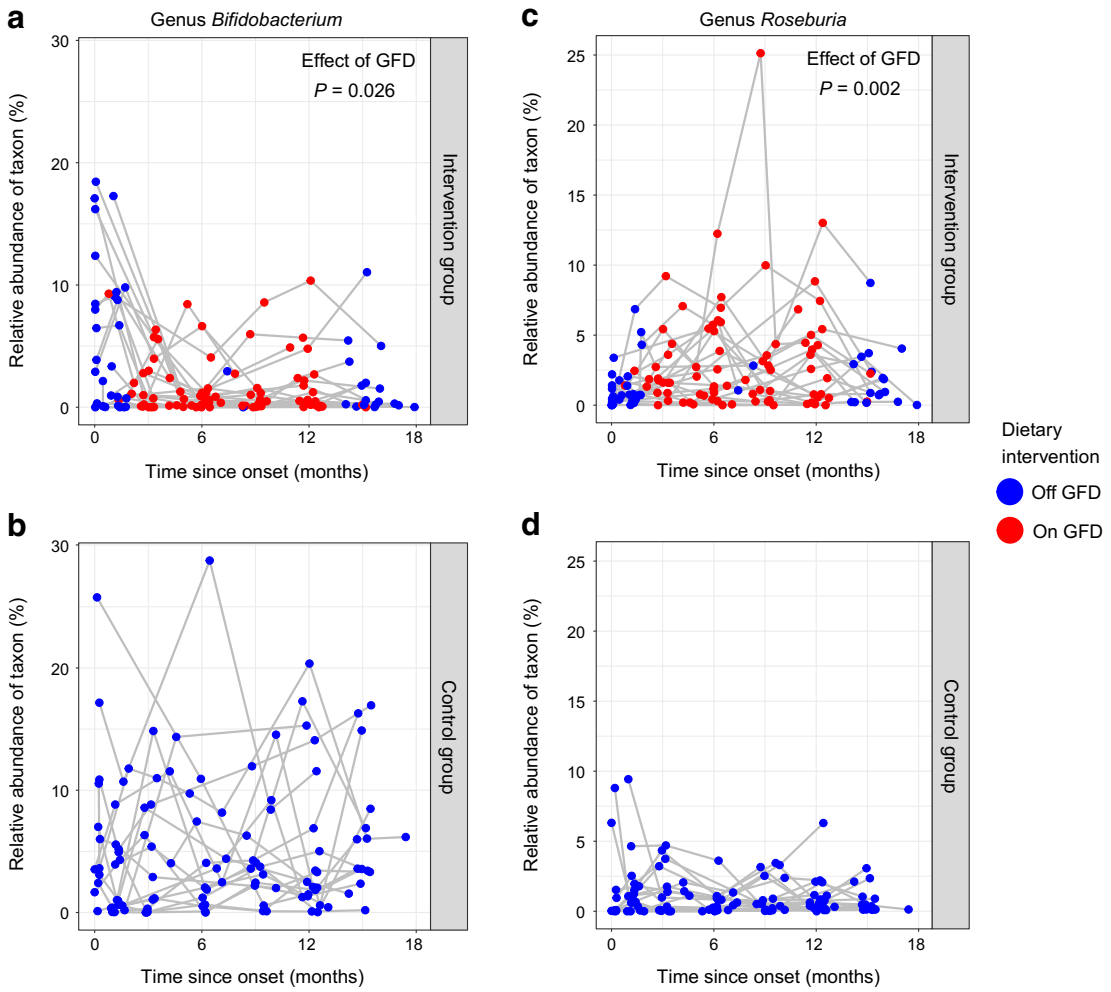

Fig. 1 Bacterial genera with significant changes in their relative abundance during the intervention with GFD. (**a**, **b**) Changes in the relative abundance of genus *Bifidobacterium* in the intervention group (**a**) and control group (**b**). (**c**, **d**) Changes in the relative abundance of genus *Roseburia* in the intervention group (**c**) and control group (**d**)

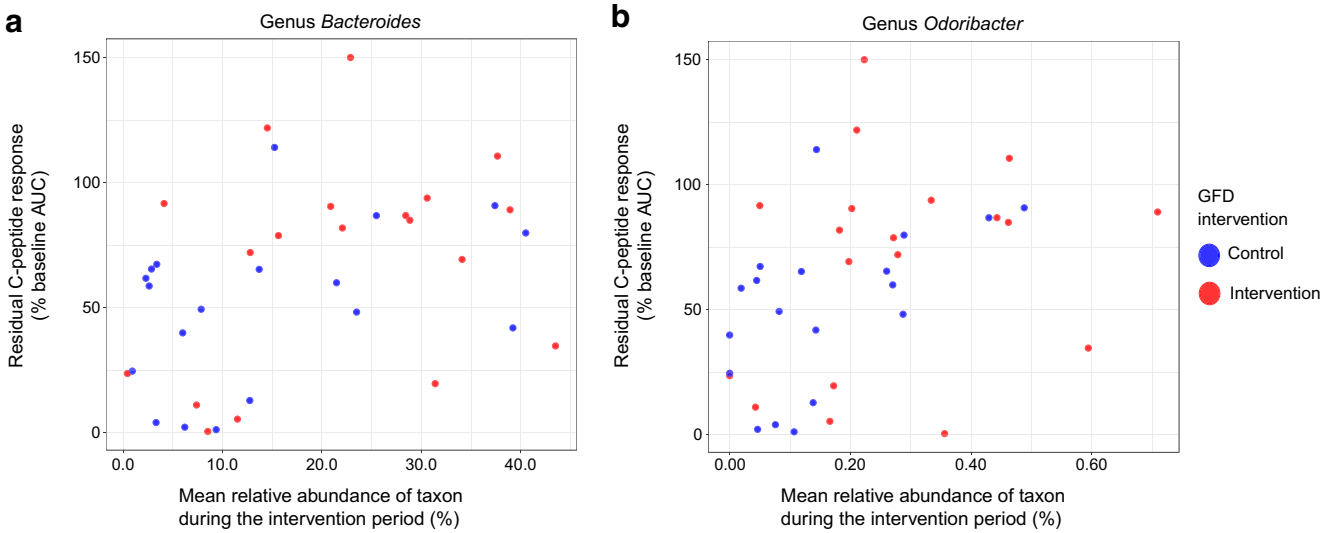

Fig. 2 Bacterial genera with the strongest direct association with beta cell residual function. (**a**) Mean relative abundance of genus *Bacteroides* against residual beta cell capacity in the intervention or control group. (**b**) Mean relative abundance of genus *Odoribacter* against residual beta cell capacity in the intervention or control group

the other metabolites normally found in stool were overlapping with glycerol, which was added as cryoprotectant. There was a highly significant correlation between the distances of sample metabolomes, and the respective distances of bacteriomes (r=0.30, p<0.001 in the Mantel test; co-inertia plots in ESM Fig. 5).

The profile of the measured metabolites did not significantly change upon the introduction of GFD (predictors fitted onto principal component analysis of Euclidean distances among the scaled metabolite levels, p=0.11 for the GFD term, adjusted for the category of time since type 1 diabetes onset). The beta cell preservation categories were not associated with the overall metabolome profiles (p=0.57), with individual metabolites, or with the potential functional capacity of the bacteriome inferred by the PICRUSt program (ESM Results 2.5).

Intestinal permeability The L/M excretion ratio at study month 12 did not differ significantly between the study groups. Similarly, no difference was noted for serum zonulin levels in any of the analysed time points (ESM Results 2.6 and ESM Fig. 6). There was no significant correlation between clinical variables or beta cell residual function and L/M excretion ratio (ESM Table 2) or zonulin levels (ESM Table 3).

Nutrient intake analysis No significant differences were observed at 6 or 12 months between the intervention and control group in energy or macronutrient intake (carbohydrates, simple carbohydrates, polysaccharides, fat, saturated fat, mono- or polyunsaturated fatty acids, cholesterol, protein and fibre) (ESM Table 4).

Discussion

The administration of the GFD in children shortly after the onset of type 1 diabetes was accompanied by subtle yet significant changes in the gut microbiome. These changes were not paralleled by alterations of the stool metabolome and were not associated with beta cell function preservation.

The reaction of the bacteriome to GFD was modest—there was no statistically significant change in the overall community composition of the bacteriome, nor were there changes in stool metabolites that largely mirror the bacterial metabolic production. Two important bacterial genera were convincingly associated with GFD intervention: *Bifidobacterium* decreased, whereas *Roseburia* increased. *Bifidobacterium* reaction to GFD has been noted in previous studies [13–15], and probably reflects its frequent ability to utilise gluten as the sole nitrogen source [16]. *Roseburia,* one of the main producers of beneficial butyrate, may increase on GFD because some gluten-free products contain high amounts of fibre and resistant starch [17], which are among the main substrates for this species [18].

The subtle microbiome changes after introducing GFD are unlikely to mediate the previously observed association between GFD and beta cell function. First, neither *Bifidobacterium* nor *Roseburia* showed association with the preservation of residual beta cell function. Moreover, no other definable taxon, metabolite or other bacteriome trait could be pinpointed as associating with the pace of loss of the residual beta cell function. Other candidate pathways should therefore be explored, be it the change in the immune landscape of the gut or the direct toxic effect of gluten peptides.

The strengths of our study lie in its longitudinal nature, as well as the length of the study period which is—to date—the longest involving individuals without coeliac disease [2, 13, 19]. Furthermore, the frequent sampling of the study participants allowed us to discard transient changes likely linked to the period around the onset of type 1 diabetes. The study also benefited greatly from an objective method of GFD adherence testing of all stool samples which is not, to our knowledge, a commonplace feature of such studies [2, 13], and rigid non-compliance criteria which led to the exclusion of four non-compliant participants. Finally, the two-step statistical analysis helped eliminate falsely positive results that might have arisen from the overly sensitive DESeq2 [20].

Among the limitations are the small study size and the rather modest association of GFD with improvements in residual beta cell function—these make any causative inference difficult. The use of the 16S rDNA marker gene approach provides less data than bacterial metagenomic or metatranscriptomic sequencing. Another limitation is the relatively low metabolite coverage of the metabolomics approach, owing to glycerol (added as part of the biobank storage protocol) overlapping with proton signals of some of the metabolites normally found in stool. Intestinal permeability marker L/M excretion ratio was obtained only at the end of the study period, thereby precluding the observation of likely transient changes, and zonulin measurements alone may not be reliable in detecting the actual protein [21], nor correlate with the gold standard sugar-absorption tests [22].

In conclusion, the bacteriome composition is unlikely to mediate the previously observed effects of GFD on beta cell residual function. This may have implications for future studies of therapeutic modalities, and underlines the need to focus on other possible mechanisms.

Supplementary Information The online version of this article (https://doi.org/10.1007/s00125-022-05805-3) contains peer-reviewed but unedited supplementary material.

Acknowledgements M. Kekrtová and L. Tylová (both Department of Nutrition, 2nd Faculty of Medicine, Charles University, Prague, Czechia) are gratefully acknowledged for the analysis of the nutritional questionnaires. O. Uhlík (Department of Biochemistry and Microbiology, University of Chemistry and Technology, Prague, Czechia) is thanked for his expert advice

on functional properties of the gut bacteriome. M. Pavlíková (Department of Probability and Mathematical Statistics, Faculty of Mathematics and Physics, Charles University, Prague, Czechia) is gratefully thanked for the statistical analysis of the data from the Czech Registry of Diabetic Children. BEI Resources (Manassas, VA, USA) are gratefully acknowledged for providing us with the Microbial Mock Community DNA (HM-276D).

Data availability All data used for the analysis in this article are available on request from the authors. The bacteriome profiling sequencing data, along with sample metadata (demographic data, beta cell residual capacity etc.), have been deposited in the NCBI Sequencing Read Archive under project number PRJNA777775.

Funding The study was funded with the support of the Ministry of Health of the Czech Republic (AZV grants 16-27994A and NU21-01-00085).

Authors' relationships and activities The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement ZS and OC conceived the study. VN acquired and analysed the data and drafted the manuscript. MK performed the data analysis and critically revised the article. ZS, SP, SK, MR, JV and LP recruited and cared for the study participants and critically revised the manuscript. SH performed the intestinal permeability analyses and participated in the revision of the manuscript. JH and AM performed the gut metabolome analyses and participated in the revision of the manuscript. OC, MK and ZS substantially contributed to the design of the study and revised the article. OC is responsible for the integrity of the work as a whole. All co-authors were given the final version of the manuscript and approved its content.

References

1. Neuman V, Pruhova S, Kulich M et al (2020) Gluten-free diet in children with recent-onset type 1 diabetes: A 12-month intervention trial. Diabetes Obes Metab 22(5):866–872. https://doi.org/10.1111/dom.13974
2. Bonder MJ, Tigchelaar EF, Cai X et al (2016) The influence of a short-term gluten-free diet on the human gut microbiome. Genome Med 8(1):45. https://doi.org/10.1186/s13073-016-0295-y
3. Lammers KM, Lu R, Brownley J et al (2008) Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. Gastroenterology 135(1):194–20 e193. https://doi.org/10.1053/j.gastro.2008.03.023
4. El Asmar R, Panigrahi P, Bamford P et al (2002) Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. Gastroenterology 123(5):1607–1615. https://doi.org/10.1053/gast.2002.36578
5. Greenbaum CJ, Mandrup-Poulsen T, McGee PF et al (2008) Mixed-meal tolerance test versus glucagon stimulation test for the assessment of beta-cell function in therapeutic trials in type 1 diabetes. Diabetes Care 31(10):1966–1971. https://doi.org/10.2337/dc07-2451
6. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 79(17):5112–5120. https://doi.org/10.1128/AEM.01043-13
7. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13(7):581–583. https://doi.org/10.1038/nmeth.3869
8. Jaimes JD, Slavickova A, Hurych J et al (2021) Stool metabolome-microbiota evaluation among children and adolescents with obesity, overweight, and normal-weight using 1H NMR and 16S rRNA gene profiling. PLoS One 16(3):e0247378. https://doi.org/10.1371/journal.pone.0247378
9. Sequeira IR, Lentle RG, Kruger MC, Hurst RD (2014) Standardising the lactulose mannitol test of gut permeability to minimise error and promote comparability. PLoS One 9(6):e99256. https://doi.org/10.1371/journal.pone.0099256
10. Fasano A, Not T, Wang W et al (2000) Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. Lancet 355(9214):1518–1519. https://doi.org/10.1016/S0140-6736(00)02169-3
11. Comino I, Fernandez-Banares F, Esteve M et al (2016) Fecal gluten peptides reveal limitations of serological tests and food questionnaires for monitoring gluten-free diet in celiac disease patients. Am J Gastroenterol 111(10):1456–1465. https://doi.org/10.1038/ajg.2016.439
12. Cuschieri S (2019) The STROBE guidelines. Saudi J Anaesth 13(Suppl 1):S31–S34. https://doi.org/10.4103/sja.SJA_543_18
13. De Palma G, Nadal I, Collado MC, Sanz Y (2009) Effects of a gluten-free diet on gut microbiota and immune function in healthy adult human subjects. Br J Nutr 102(8):1154–1160. https://doi.org/10.1017/S0007114509371767
14. Golfetto L, de Senna FD, Hermes J, Beserra BT, Franca Fda S, Martinello F (2014) Lower bifidobacteria counts in adult patients with celiac disease on a gluten-free diet. Arq Gastroenterol 51(2):139–143. https://doi.org/10.1590/s0004-28032014000200013
15. Di Cagno R, De Angelis M, De Pasquale I et al (2011) Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization. BMC Microbiol 11(1):219. https://doi.org/10.1186/1471-2180-11-219
16. Caminero A, Herran AR, Nistal E et al (2014) Diversity of the cultivable human gut microbiome involved in gluten metabolism: isolation of microorganisms with potential interest for coeliac disease. FEMS Microbiol Ecol 88(2):309–319. https://doi.org/10.1111/1574-6941.12295
17. Larretxi I, Churruca I, Navarro V et al (2020) Effect of analytically measured fiber and resistant starch from gluten-free products on the diets of individuals with celiac disease. Nutrition 70:110586. https://doi.org/10.1016/j.nut.2019.110586
18. Walker AW, Ince J, Duncan SH et al (2011) Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J 5(2):220–230. https://doi.org/10.1038/ismej.2010.118
19. Hansen LBS, Roager HM, Sondertoft NB et al (2018) A low-gluten diet induces changes in the intestinal microbiome of healthy Danish adults. Nat Commun 9(1):4630. https://doi.org/10.1038/s41467-018-07019-x
20. Thorsen J, Brejnrod A, Mortensen M et al (2016) Large-scale benchmarking reveals false discoveries and count transformation sensitivity in 16S rRNA gene amplicon data analysis methods used in microbiome studies. Microbiome 4(1):62. https://doi.org/10.1186/s40168-016-0208-8
21. Ajamian M, Steer D, Rosella G, Gibson PR (2019) Serum zonulin as a marker of intestinal mucosal barrier function: May not be what it seems. PLoS One 14(1):e0210728. https://doi.org/10.1371/journal.pone.0210728
22. Tatucu-Babet OA, Forsyth A, Owen E et al (2020) Serum zonulin measured by enzyme-linked immunosorbent assay may not be a reliable marker of small intestinal permeability in healthy adults. Nutr Res 78:82–92. https://doi.org/10.1016/j.nutres.2020.05.003