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Gut microbial clusters in children show different cardiometabolic responses to wholegrains – a *post hoc* analysis of a randomized wholegrain trial in children

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The beneficial effects of wholegrain intake on cardiometabolic risk may be mediated by the gut microbiota. In adults, cardiometabolic responses appear to be influenced partly by the structure of the gut microbiome ecosystem. However, this has not been investigated in children. We aimed to identify gut microbial clusters among children and explore their role in the cardiometabolic response to high wholegrain intake. The present study utilized data from a previous randomized cross-over study involving 51 Danish 8–13 year-old children with high BMI, provided with wholegrain oats and rye ("WG") and refined grain ("RG") for 8 weeks in random order. Anthropometry, body composition, cardiometabolic markers in blood, and blood and faecal short-chain fatty acids were assessed at 0, 8 and 16 weeks. Faecal microbiota composition was assessed by 16S rRNA gene amplicon sequencing and microbial clusters were identified by partitioning around medoid clustering. We used linear mixed models to investigate the modifying effects of the baseline microbial cluster on the cardiometabolic effects of wholegrain. We identified two clusters dominated by *Faecalibacterium* (Cluster 1, n = 25), and *Clostridium* (Cluster 2, n = 26), respectively. Cluster modified the effects of WG on BMI z-score, fat mass index, and waist circumference (all $P_{interaction} \leq 0.002$), which were reduced among children in Cluster 1 and increased in those in Cluster 2. Cluster also modified the effect on HDL cholesterol ($P_{interaction}$ = 0.03) and tended to modify the effect on faecal butyrate. In conclusion, two distinct gut microbial clusters were identified in schoolchildren, which appeared to affect how effectively a high wholegrain intake reduced fat mass.

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Introduction

Wholegrain intake has consistently been inversely associated with type 2 diabetes (T2D),[1] cardiovascular disease (CVD),[2] and weight gain[3,4] in observational studies. Randomized trials, particularly investigating wholegrain oat and rye intake, show beneficial effects on cardiometabolic risk markers in adults,[5–12] and emerging research suggests similar effects among children.[13–17] The cardiometabolic benefits of wholegrain intake have been suggested to be mediated by fermentation of cereal fibres by the gut microbiota, leading to production of short-chain fatty acids (SCFA),[10,12,17–20] which have demonstrated beneficial metabolic effects in humans and animal models.[21–24] However, the overall evidence of the effects of wholegrain is inconclusive due to large between-study heterogeneity.[25] While variations in the results of trials may partly be attributed to the administration of various wholegrain types, individualized responses to wholegrain intake may also play a role, as explorative studies have shown that the effects of wholegrain consumption on cardiometabolic risk markers in adults can be modified by baseline gut microbial composition.[10,26–28]

The establishment of stable gut microbial community structures and clusters, also referred to as "enterotypes", has traditionally been recognized to occur within the first few years of life, influenced by factors such as birth mode, breastfeeding, and number of siblings.[29] Emerging research has challenged this understanding, revealing continued gut microbial devel-

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opment throughout mid-childhood, partly influenced by habitual diet, in particular dietary fibre intake,[30–33] but gut microbial clusters have yet to be thoroughly investigated across diverse young populations during the transition into adulthood. Three robust cross-national enterotypes, characterized by the variation in the abundances of *Bacteroides*, *Prevotella*, and *Ruminococcus*, are typically identified in adults.[34,35] Enterotypes with high abundance of *Prevotella* and *Bacteroides* have been linked to long-term dietary patterns in adults[36] and the ratio between these two taxa has demonstrated stability even following an intervention diet rich in dietary fibre.[37] Noticeably, previous studies have shown that diets high in wholegrain or dietary fibre can have beneficial effects on weight loss,[26,27] glucose metabolism[28] and blood lipids[10] among adults with a high *Prevotella* abundance. However, the potential modifying effect of pre-treatment gut microbiota on cardiometabolic risk markers has never been explored in dietary intervention trials among children.

The present exploratory study was based on the randomized cross-over trial, KORN, among Danish 8–13 year-olds with a high body mass index (BMI).[17] The study showed that high intake of wholegrain oats and rye ("WG"), compared to refined grains ("RG"), reduced serum low-density lipoprotein cholesterol (LDL-C), modulated the relative abundance of specific microbial taxa, and increased SCFA in both plasma and faecal samples. In the present study, we aimed to identify gut microbial clusters present before the intervention and investigate whether these clusters modify the cardiometabolic response to wholegrain intake in schoolchildren.

Experimental

Study design, participants and randomization

The present study used data from a 2 × 8 week randomized crossover study among children that was conducted at the Department of Nutrition, Exercise and Sports (NEXS), University of Copenhagen, Denmark during September 2020 to May 2021.[17] The study protocol was approved by the Committees on Biomedical Research Ethics for the Capital Region of Denmark (no. H-19010737) and registered with clinicaltrials.gov (NCT04430465, Study Record | Beta ClinicalTrials.gov). Written consent was obtained from all custody holders of the children prior to enrolment.

Eligible children living in the Capital Region of Denmark were identified through the national civil registry and invited by digital invitation to custody holders. In order to be included in the trial, the children had to be 8–13 years old, have a reported BMI >1 SD above the median according to sex and age-standardized Danish growth curves,[17] eat grain products daily, speak Danish, and have at least one parent fluent in Danish. Exclusion applied for children with allergies to the study products, the use of dietary fibre or probiotic supplements, energy restricted diets, serious illness, medication that could interfere with the intervention, participation in other trials or cohabitation with another participating child.[17]

Children were randomized to a treatment sequence in blocks of six at the end of the baseline examination visit. Study participants and staff were not blinded during the intervention, but investigators were blinded during laboratory analysis. The study was conducted during the COVID-19 pandemic, which involved lockdown of schools and sports activities from December 9, 2020, to February 8, 2021, so children were home-schooled during this period.

We included 55 children in the trial; 52 of them completed the intervention and the 51 children who had available baseline microbiota data were included in the present exploratory study.

Study products

Children were provided with study products containing wholegrain from oats and rye, "WG", and refined grain, "RG", for 2 eight week periods, in random order. The products included breakfast cereals, breads, pasta, *etc*., and were provided *ad libitum* to substitute for the grain products habitually eaten by the children during a whole day as previously described.[17] Children consumed 108 ± 38 and 3 ± 2 g d^{-1} wholegrain in the WG and RG periods, respectively, which was verified by a profound between-period difference in plasma alkylresorcinols ($P < 0.001$).[17]

Questionnaires

Questionnaires about the household's highest education, children's pubertal stage, physical activity and screen time were completed online within one week prior to the first visit as previously described.[17]

Dietary intake

Dietary intake was recorded by the parents with help from the children during the last week prior to each examination visit, using a 4-day weighted dietary record on three consecutive weekdays and one weekend day as previously described.[17] Intake of study products was included in the 4-day dietary records, but the reported daily intake was based on daily self-recording of the consumed study product (in decilitres or grams) throughout the trial duration.

Anthropometry, body composition and blood pressure

At 0, 8 and 16 weeks, we measured the children's height, weight, waist circumference, blood pressure, and body composition by whole body DXA, as detailed.[17] Age- and sex-adjusted *z*-scores for BMI were calculated based on the WHO's growth standards using the WHO 2007 R macro package, WHO2007_R.zip.[38] The fat mass index (FMI) was calculated as kg of total body fat divided by the squared height in m.

Blood and faecal samples

At each time point, we collected fasting blood samples which were processed and analysed according to specific protocols as previously described.[17] We quantified serum LDL-C, high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and triglycerides (TG), plasma insulin and glucose, serum

C-reactive protein (CRP) and interleukin 6 (IL-6) and plasma SCFA, including acetate, propionate and butyrate.

Faecal samples were collected and analysed for concentrations of acetate, propionate and butyrate using liquid chromatography with tandem mass spectrometry (LC-MS/MS) as previously described.[17] The gut microbiota composition was determined by 16S rRNA gene amplicon sequencing (Illumina NextSeq). Details of DNA extraction, library preparation, sequencing and construction of the amplicon sequencing variant (ASV) table have been provided previously.[39] Phylogenetic annotation of the gut microbiome including agglomeration to the genus level was performed using previously described procedures.[39] The microbiota composition was classified using Jensen–Shannon distance and partitioning around medoid (PAM) clustering.[35] The Calinski–Harabasz (CH) index was applied to determine the optimal number of clusters and the silhouette index was calculated using R.[40] We tested for significant differences between clusters using PERMANOVA (999 permutations) with the vegan R package (v2.6-2).

Statistical analyses

Descriptive data are presented as mean ± SD or median (25^{th}–75^{th} percentiles) for normally distributed and skewed variables, respectively. Background characteristics, dietary intake and cardiometabolic markers were compared between Cluster 1 and Cluster 2, using unpaired Student's *t*-test or Wilcoxon rank-sum test, for normally distributed and skewed continuous variables, respectively, and Pearson's chi-squared test for categorical data. The relative abundances of the top 15 genera were compared between clusters, using a non-parametric test with *p* values adjusted using Holm–Bonferroni false discovery rate correction.

To investigate the effects of WG compared to RG on cardiometabolic risk markers and SCFAs and the involvement of microbial clusters, we used linear mixed models with a treatment × cluster interaction. In the full model, fixed effects included treatment (WG/RG), cluster (1/2), the treatment × cluster interaction term (4 levels), baseline value and homeschooling due to COVID-19 (% of all days), while the subject was modelled as a random effect to adjust for within-subject correlation. Treatment effects were analysed as mean differences (95% CI) between WG and RG within each microbial cluster and potential treatment modification by cluster was analysed using an *F*-test, comparing the full model with a similar model without the interaction term. Models were verified based on residual and normal probability plots. Insulin, TG, CRP and IL-6 were log-transformed prior to analyses, and estimates were back-transformed, as previously described.[41]

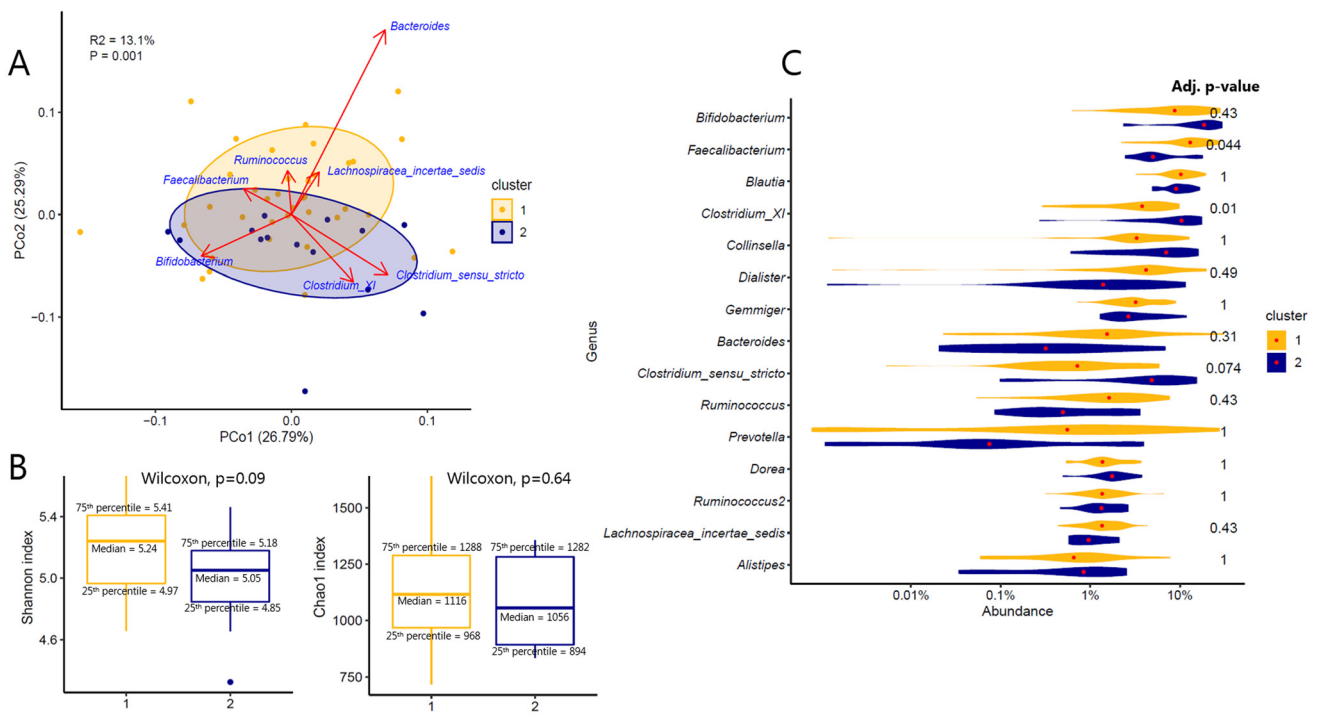

Fig. 1 (A) At the baseline, the children from the KORN study (n = 51) separated into two microbial clusters (Cluster 1 and Cluster 2) using the partitioning around medoids algorithm. The between cluster *P*-value is based on comparison using PERMANOVA (999 permutations). (B) α-Diversity within the two microbial clusters defined by the Shannon index and Chao1 index. Data are given as median (25th–75th percentiles) and *P*-values are based on the Wilcoxon rank sum test comparisons between the clusters. (C) Distribution of the top 15 generas' relative abundances in the two clusters. The red dots represent median values plotted on a log10 scaled *x*-axis. Adjusted *p*-values for between cluster comparisons of relative abundance were determined by the non-parametric test with Holm–Bonferroni false discovery rate correction.

All statistical analyses were performed in R version 4.2.1, using particularly the lmerTest package version 3.1-3.[42] Statistical significance was established at $P < 0.05$.

Results and discussion

The 51 included children were on average 11 years of age and had a BMI *z*-score of 1.5 ± 0.6 (mean ± SD); 47% were females. The children were separated into two distinct gut microbial clusters by Jensen–Shannon distance and PAM clustering with 25 children in Cluster 1 and 26 children in Cluster 2 (Fig. 1A). The average silhouette index of the clusters was 0.138, indicating that the 2 clusters separate, but with the clustering of some subjects being associated with some uncertainty as also evident by visual inspection. However, importantly, the allocation to the two clusters remained robust after the start of the intervention (chi-squared test for comparison of cluster allocation at the baseline and first visit, $P = 0.114$). The children in the two clusters did not differ in any of the assessed variables at the baseline, although LDL cholesterol tended to be higher in Cluster 2 and dietary fibre intake tended to be higher in Cluster 1 (Table 1). Cluster 1 tended to have higher diversity than Cluster 2, as determined by the Shannon diversity index ($P = 0.09$), but clusters did not differ in species richness as determined by the Chao1 index (Fig. 1B). Following adjustment for multiple testing, the children in Cluster 1 showed a higher relative abundance of *Faecalibacterium*, whereas those in Cluster 2 had higher relative abundance of *Clostridium* cluster XI and *Clostridium sensu stricto* (Fig. 1C).

The effect of WG *versus* RG on BMI *z*-scores, FMI, waist circumference ($P_{interaction} \leq 0.002$) and HDL cholesterol ($P_{interaction} = 0.030$) differed between Cluster 1 and Cluster 2 (Table 2). All measures of obesity decreased among the children in Cluster 1 following WG intervention, while the children in Cluster 2 exhibited increases in the adiposity measures and increasing HDL cholesterol (Table 2 and Fig. 2). A significant increase in faecal butyrate with WG compared to RG was only observed for children in Cluster 1 and this was supported by a tendency of effect modification by cluster ($P_{interaction} = 0.09$) (Table 2). In concurrence, children in Cluster 1 showed increased plasma SCFAs (acetate, propionate and butyrate) following the WG intervention, whereas this was not observed in Cluster 2 (Table 2).

Table 1 Baseline characteristics by microbial cluster (mean ± SD)

	Cluster 1 ($n = 25$)	Cluster 2 ($n = 26$)	*P*-Value[a]
Female sex (*n*, %)	13, 52	11, 42	0.680
Age (years)	11.1 ± 1.8	11.1 ± 1.9	0.969
Tanner stage (>1) (*n*, %)	15, 60	15, 58	1.000
Body weight (kg)	50.1 ± 11.3	49.8 ± 11.8	0.931
BMI (*z*-score)	1.4 ± 0.6	1.6 ± 0.6	0.408
Fat mass index (kg m^{-2})	8.0 ± 2.2	8.4 ± 1.8	0.574
Waist circumference (cm)	78.2 ± 8.8	77.7 ± 9.0	0.852
Diastolic blood pressure (mmHg)	65 ± 3	66 ± 4	0.249
Systolic blood pressure (mmHg)	103 ± 5	104 ± 6	0.304
LDL cholesterol (mmol L^{-1})	2.1 ± 0.5	2.5 ± 0.7	0.060
HDL cholesterol (mmol L^{-1})	1.4 ± 0.2	1.3 ± 0.2	0.500
Total cholesterol (mmol L^{-1})	3.8 ± 0.6	4.1 ± 0.8	0.169
Total: HDL cholesterol	2.9 ± 0.6	3.2 ± 0.7	0.130
Triacylglycerol (mmol L^{-1})[b]	0.58 (0.48–0.93)	0.65 (0.51–0.85)	0.547
Insulin (pmol L^{-1})[b]	59 (49–82)	73 (50–87)	0.322
Glucose (mmol L^{-1})	5.1 ± 0.4	5.1 ± 0.4	0.428
HOMA-IR[b]	2.2 (1.7–3.2)	2.8 (2.0–3.4)	0.320
C-reactive protein (mg L^{-1})[b]	0.35 (0.21–0.67)	0.44 (0.21–0.82)	0.534
IL-6 (pmol L^{-1})[b]	0.94 (0.59–1.17)	0.91 (0.61–1.54)	0.698
Plasma acetate (μmol L^{-1})	88 ± 60	95 ± 76	0.690
Plasma propionate (μmol L^{-1})	1.0 ± 0.5	1.1 ± 0.5	0.499
Plasma butyrate (μmol L^{-1})	0.8 ± 0.6	0.9 ± 0.8	0.679
Fecal acetate (μmol g^{-1})	161 ± 84	151 ± 59	0.654
Fecal propionate (μmol g^{-1})	50 ± 22	57 ± 38	0.457
Fecal butyrate (μmol g^{-1})	65 ± 36	54 ± 25	0.190
MVPA (min d^{-1})[b]	60 (41–146)	66 (36–136)	0.828
Screen time (min d^{-1})[b]	60 (30–97)	56 (27–133)	0.748
Education ≥master's degree (*n*, %)[c]	11, 44	12, 46	1.000
Total energy intake (MJ per day)	7.9 ± 1.8	7.8 ± 1.9	0.886
Dietary fat intake (*E*%)	33 ± 6	31 ± 4	0.219
Dietary protein intake (*E*%)	16 ± 2	15 ± 2	0.390
Dietary carbohydrate intake (*E*%)	51 ± 6	54 ± 4	0.127
Dietary fiber intake (g d^{-1})	20.6 ± 7.0	17.0 ± 7.3	0.080

$n = 24$ for all blood measurements in Cluster 1. MVPA, moderate to vigorous physical activity. [a] *P*-Values for comparison of Clusters 1 and 2 using Student's *t*-test, Wilcoxon rank-sum test and Pearson's chi-squared test as appropriate. [b] Median (25^{th}–75^{th} percentiles). [c] Household's longest education.

Table 2 Estimated treatment effects (mean difference, 95% CI) in body composition, cardiometabolic risk markers and SCFAs stratified by microbial cluster and test for interaction between the treatment and microbial cluster

		Cluster 1		Cluster 2		
		WG–RG[a]	*P*-Value[a]	WG–RG[a]	*P*-Value[a]	Treatment × cluster *P*-Value[b]
Anthropometry	BMI (*z*-score)	−0.06 (−0.11, −0.01)	**0.022**	0.06 (0.01, 0.11)	**0.04**	**0.002**
	Fat mass index (kg m^{-2})	−0.30 (−0.48, −0.12)	**0.002**	0.24 (0.07, 0.42)	**0.01**	**<0.001**
	Waist circumference (cm)	−0.89 (−1.73, −0.06)	**0.041**	1.21 (0.39, 2.03)	**0.006**	**<0.001**
Blood pressure	Diastolic blood pressure (mmHg)	−0.25 (−1.52, 1.01)	0.696	−0.53 (−1.77, 0.71)	0.410	0.758
	Systolic blood pressure (mmHg)	−0.37 (−2.11, 1.36)	0.675	−1.321 (−3.02, 0.38)	0.134	0.437
Blood lipids	LDL cholesterol (mmol L^{-1})	−0.09 (−0.23, 0.06)	0.240	−0.19 (−0.33, −0.04)	**0.013**	0.330
	HDL cholesterol (mmol L^{-1})	−0.01 (−0.08, 0.05)	0.711	0.09 (0.03, 0.15)	**0.009**	**0.030**
	Total cholesterol (mmol L^{-1})	−0.18 (−0.37, 0.02)	0.084	−0.09 (−0.28, 0.11)	0.397	0.504
	Total:HDL cholesterol	−0.13 (−0.28, 0.02)	0.104	−0.28 (−0.43, −0.13)	**<0.001**	0.158
	Triacylglycerol (mmol L^{-1})[c]	−0.06 (−0.21, 0.08)	0.377	−0.15 (−0.28, −0.01)	0.045	0.411
Glucose metabolism	Insulin (pmol L^{-1})[c]	2.32 (−11.65, 16.28)	0.747	−4.09 (−17.84, 9.66)	0.563	0.514
	Glucose (mmol L^{-1})	−0.03 (−0.16, 0.10)	0.643	−0.08 (−0.21, 0.05)	0.213	0.567
Inflammatory markers	C-reactive protein (mg L^{-1})[c]	−0.14 (−0.33, 0.05)	0.154	−0.13 (−0.32, 0.06)	0.178	0.964
	Interleukin-6 (pmol L^{-1})[c]	0.08 (−0.16, 0.32)	0.526	−0.11 (−0.35, 0.12)	0.352	0.257
Short-chain fatty acids	Plasma acetate (μmol L^{-1})	41 (11, 70)	**0.008**	20 (−10, 49)	0.196	0.297
	Plasma propionate (μmol L^{-1})	0.26 (0.06, 0.46)	**0.015**	0.04 (−0.16, 0.24)	0.698	0.134
	Plasma butyrate (μmol L^{-1})	0.42 (0.14, 0.70)	**0.004**	0.26 (−0.02, 0.54)	0.067	0.417
	Fecal acetate (μmol g^{-1})	13 (−26, 52)	0.524	−2.1 (−41, 37)	0.916	0.588
	Fecal propionate (μmol g^{-1})	0 (−11, 11)	0.995	−7 (−18, 3)	0.197	0.350
	Fecal butyrate (μmol g^{-1})	27 (12, 42)	**<0.001**	9 (−5, 24)	0.220	0.090

Bold indicates *p*-values of 0.05 or below. $N = 51$ for all analysis. RG, refined grain; WG, wholegrain. [a] Estimates and *p*-values from mixed model with treatment × cluster interaction, baseline value and homeschooling as fixed effects and subject as random effect. [b] *P*-Values from *F*-test comparing model with treatment × cluster interaction, baseline value and homeschooling as fixed effects and subject as random effect with a similar model without an interaction term. [c] Median (25^{th}–75^{th} percentiles). Model estimates and confidence intervals were back-transformed to their original scale.

Among Danish school children aged 8–13 with high BMI, we identified two gut microbial clusters that remained stable after the start of the intervention. The clustering appeared to be driven by taxa indicative of a shift between two groups of SCFA-producing bacteria. Cluster 1 was characterized by higher relative abundance of the butyrate-producing *Faecalibacterium* whereas Cluster 2 tended to be less diverse and was dominated by higher relative abundance of *Clostridium* cluster XI and *Clostridium sensu stricto*. Intriguingly, the identified clusters modified the effect of wholegrain on BMI, FMI, and waist circumference as only children in Cluster 1 had reduced adiposity measures in response to WG. Furthermore, cluster modified the effect of WG on HDL cholesterol, with raised concentrations following WG among children in Cluster 2 only. Together, these results suggest that differences in microbiome structures could affect metabolic responses to high wholegrain intake in children.

A previous study of 281 Dutch 6–10-year-olds found that the children could be divided into three clusters or enterotypes dominated by *Bifidobacterium*, *Bacteroides* and *Prevotella*, respectively. The *Bifidobacterium* enterotype was characterized by lower α-diversity compared to other clusters.[30] In the present study, which focused on slightly older children with a high habitual intake of wholegrains, we identified only two distinct clusters. The lower alpha-diversity and increased abundance of *Clostridium sensu stricto* in Cluster 2 could reflect an immature and less adult-like gut microbiota, since this taxon comprises a pronounced butyrate-producing member of the infant gut microbiota.[43] Cluster 1 showed higher levels of *Faecalibacterium*, which is a prominent butyrate-producing taxon that increases with age[43] and pre-dominates the adult gut microbiota.[44,45] While no age difference was observed between clusters, children in Cluster 1 showed a tendency of higher dietary fibre intake. Dietary fibre has been shown to be a key factor in the transition towards a more mature adult-like microbiota,[46] which rather than reaching a stable state around 3 years of age as previously believed, may continue to develop into childhood and adolescence.[29,47]

The reduction in body fatness in response to WG among children in Cluster 1 is in line with data from adults, where gut microbial enterotypes favouring the dietary fibre-associated taxa, *e.g. Prevotella*, have been shown to predict wholegrain induced weight loss.[26,27] Similarly, more mature gut microbiota compositions with a high dietary fibre-degrading potential have been inversely associated with markers of insulin resistance in both Dutch[30] and Mexican children.[48] Although in the original study, butyrate was linked to LDL cholesterol, the reduction in body fatness in Cluster 1 in the present analysis may be due to the higher butyrate-producing capacity of *Faecalibacterium* relative to the capacity of *Clostridium sensu stricto*.[43] Although we found no significant difference in faecal butyrate between clusters at the baseline, there was a trend of effect modification on faecal butyrate in the same direction as the obesity measures. Furthermore, significant changes in both plasma butyrate and faecal butyrate were observed in Cluster 1, suggesting that these children par-

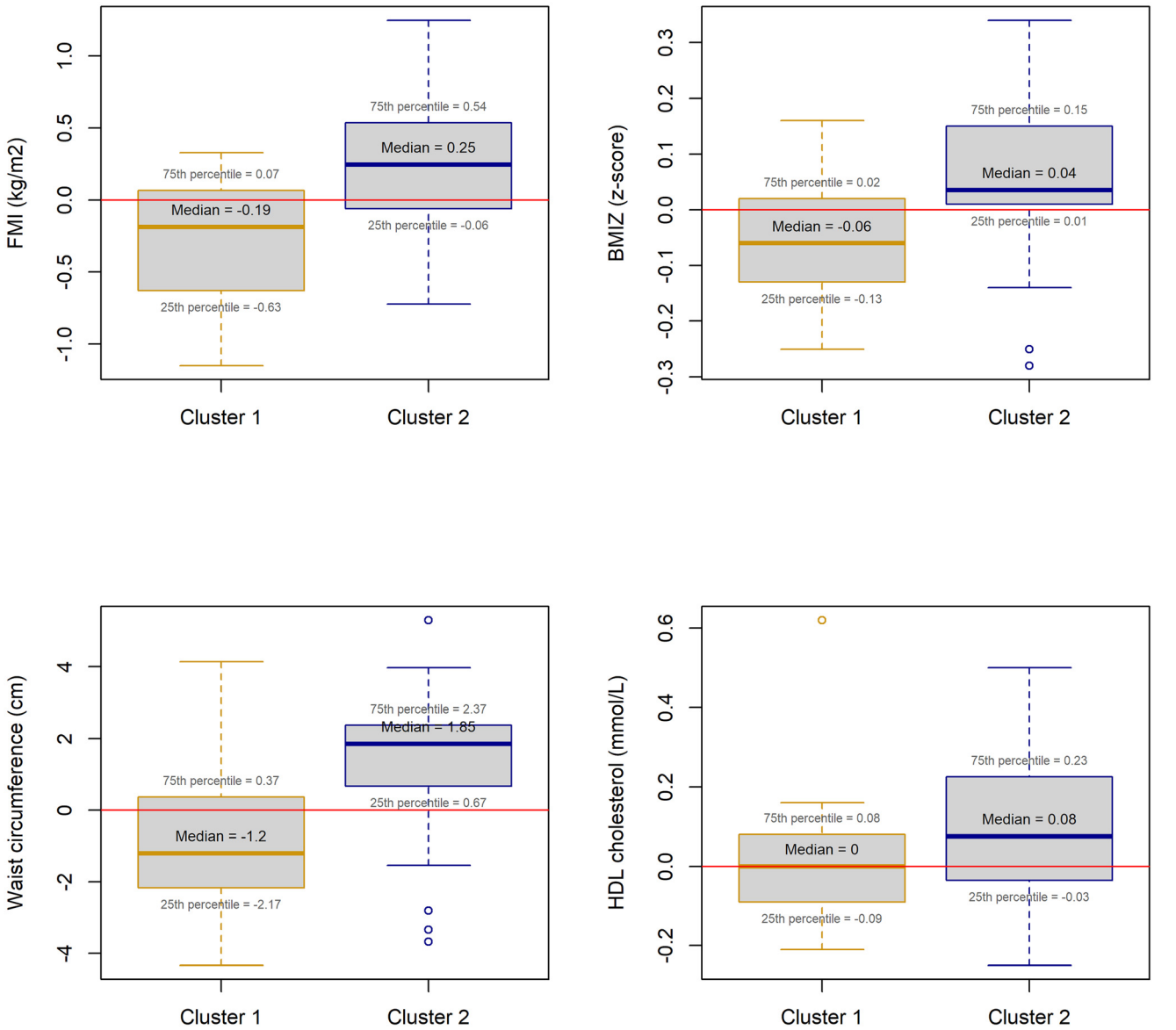

Fig. 2 Difference in adiposity measures and HDL cholesterol with wholegrain *versus* refined grain intervention stratified for Cluster 1 and Cluster 2. Differences are shown as median (25th–75th percentiles) wholegrain response relative to the response to refined grain with a red no-difference reference line.

ticularly responded to the intervention. Butyrate has been linked to induced thermogenesis, fat oxidation,[49] and release of appetite-regulating hormones in response to wholegrains.[50] Moreover, findings from a 6-month clinical trial in children with obesity suggest that oral butyrate supplementation may lower BMI and have beneficial effects on glucose metabolism and inflammation.[51] However, this warrants further investigation in mechanistic studies or studies with better power for stratified analyses. Combined with previous findings, our results suggest that some beneficial responses to wholegrains may be more pronounced in children with a more mature gut microbiota composition, likely due a greater capacity to degrade dietary fibres. Notably, we observed an increased relative abundance of *Faecalibacterium* in response to WG in the original study,[17] highlighting the potential of high wholegrain consumption to promote gut microbiome maturation towards a more metabolically advantageous composition during mid-childhood.

In contrast, children in Cluster 2 showed improved HDL cholesterol in response to WG. Noticeably, children in Cluster 2 had higher LDL cholesterol at the baseline compared to children in Cluster 1, which might pre-dispose this group to a greater potential for improvements in the lipid profile in response to the WG treatment, as previously observed in wholegrain trials.[9]

The study had a very low drop-out rate and high compliance based on self-reported intake and plasma biomarkers of WG intake. The cross-over design is a strength since it can efficien-

tly be used to compare relatively small treatment effects by eliminating any between-individual variance due to differences in genetics and background diets. Another strength of our study is the use of gold standard outcome measurements such as DXA scans, fasting blood samples and robust faecal microbiome analysis. Children up to 4–6 years of age have previously been shown to exhibit greater microbial plasticity than adults, indicating that enterotypes may not remain stable during a dietary intervention.[52] Nevertheless, the observed microbial clusters remained consistent after initiating the grain intervention. This apparent stability in the present study may be because most participants were aged 8–13 years, a period beyond the early developmental window typically associated with enterotype establishment. Alternatively, the relatively short duration of the intervention was insufficient to induce a shift in enterotype composition. However, even a 6-month dietary intervention with more fruits, vegetables and wholegrains was insufficient to alter enterotypes in adults.[37] The interaction between treatment and baseline gut microbial clusters may introduce residual confounding in the analyses due to other host traits associated with these clusters, such as unmeasured clinical baseline values. As the trial was not primarily designed for microbial cluster analysis, the reduced statistical power resulting from data stratification and potential interaction with baseline microbiota composition may partly explain the attenuation or loss of some wholegrain intervention effects, observed in the original study,[17] in this stratified analysis. Finally, there is a risk of false positive findings, which was partly mitigated through conservative correction for multiple testing in the microbiota data analyses. Moreover, the consistent direction of adiposity outcomes supports the validity of the current findings.

Conclusions

In conclusion, this exploratory study identified two distinct gut microbial clusters among Danish children aged 8–13 years with high BMI. These clusters appeared to modify the effects of wholegrain intervention, favouring children with relatively high levels of *Faecalibacterium* for reducing fat mass, and those enriched with taxa within the *Clostridiales* order for increasing HDL-C. While the findings need to be validated to establish causality, the observations are intriguing and provide a rationale for investigating the possible pivotal role of gut microbiome maturation during childhood in diet-induced cardiometabolic responses.

Author contributions

M. B. M. M. helped plan and conducted the study, analysed data, wrote the paper and had primary responsibility for the final content. C. T. D. and R. L. designed the study. D. S. N. and Y. Z. carried out gut microbiota characterization and bioinformatics. C. T. D., D. S. N., H. M. R., L. C., and L. L. contributed with writing the manuscript and the interpretation of results. All authors read and approved the final manuscript.

Conflicts of interest

None of the authors reported a conflict of interest related to the study.

Abbreviations

AR	Alkylresorcinols
B	Baseline
FMI	Fat mass index
RG	Refined grain
WG	Wholegrain

Data availability

Data for the article is collected from human participants and cannot be made available due to legal and ethical confidentiality requirements.

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