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A randomized controlled trial of environmental richness on gastrointestinal symptoms, salivary cortisol, and gut microbiota in early childhood

Chikako Shibata[1,2]✉, Tomohiko Muratsubaki[1,7], Suguru Shibata[3], Emiko Aizawa[1,4], Satoshi Watanabe[1], Motoyori Kanazawa[1,7] & Shin Fukudo[1,5,6,7]

Gastrointestinal (GI) symptoms are common and can affect children's social lives. This study investigated the effects of exposure to a rich natural environment on GI symptoms, salivary cortisol levels, salivary amylase levels, and the gut microbiota in young children. Children aged 5–6 years from four kindergartens in Japan were randomly assigned to two groups: a nature childcare group and a regular childcare group. The children were exposed to their respective conditions once weekly for one month. Before and after the intervention, GI symptoms were detected using the Children's Somatization Inventory to calculate a 'GI score' and categorize participants into GI and control groups (primary outcome measure). Fecal examinations were performed for gut microbiota using 16 S-rRNA analysis, salivary cortisol and amylase levels were quantified, and the Child Behavior Checklist was administered. The two groups had similar GI symptoms, salivary cortisol and amylase levels, and behavioral characteristics. Following the intervention, significant differences in the GI score, abdominal pain, constipation, Shannon index value, and salivary cortisol and amylase levels ($p < 0.05$) were observed between the two childcare groups. Spending free and abundant time in nature during early childhood could help maintain digestive system homeostasis, increase gut microbiota diversity, and reduce cortisol levels.

Gastrointestinal symptoms are common and can influence the social life of young children[1]. Irritable bowel syndrome (IBS) can cause recurrent abdominal pain, and the symptoms associated with IBS can lead to early social maladjustment, which can manifest in several ways, including refusal to go to school. Gastrointestinal symptoms affect up to 40% of the general population worldwide[2]. Thus, gastrointestinal symptoms are prevalent, and focusing on these symptoms is essential. Although precise data on the prevalence of gastrointestinal symptoms in young children are unavailable, a previous study from our laboratory reported a 58% prevalence of gastrointestinal symptoms in seven-year-old children[3]. Gastrointestinal symptoms thus appear to be at least as common in children as in adults. These symptoms are associated with stress responses, cortisol, amylase, and gut bacteria[4].

Regarding stress responses, animal models and clinical populations provide evidence that early life stressors can lead to an aberrant response of the hypothalamic-pituitary-adrenocortical (HPA) axis and the arousal/sympathetic nervous system and increase the incidence of stress-related disorders after puberty[5,6]. Salivary amylase and cortisol are two widely used proxy measures of stress[7,8], and increased baseline salivary cortisol levels have been observed in patients with IBS relative to controls[7]. A dysregulated innate immune system has been suggested to result from childhood trauma and lead to an increased vulnerability to psychopathology

[1]Department of Behavioral Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryo-Machi, Aoba-Ku, Sendai 980-8575, Miyagi, Japan. [2]Department of Exercise Education for Children, Faculty of Sports Science, Sendai University, Sendai, Japan. [3]Department of Early Childhood Education, Koriyama Women's University Junior College, Koriyama, Japan. [4]Department of Health and Nutrition, Faculty of Human Sciences, Sendai Shirayuri Women's College, Sendai, Japan. [5] Research Center for Accelerator and Radioisotope Science, Tohoku University, Sendai, Japan. [6] Department of Psychosomatic Medicine, Japanese Red Cross Ishinomaki Hospital, Ishinomaki, Japan. [7] Department of Psychosomatic Medicine, Tohoku University Hospital, Sendai, Japan. ✉email: ck-shibata@sendai-u.ac.jp

and somatic disorders across the lifespan[9]. A body of evidence also suggests that sleep disturbances associated with childhood adversity have a causal role in elevating an individual's vulnerability to stress in adulthood[10]. Responsive and predictable caregiver behaviors can improve child outcomes[11]. If early environmental stressors lead to stress vulnerabilities later in life, the question that naturally follows is whether enrichment of a child's psychosocial environment can reduce the risk of such stress vulnerabilities in adulthood.

The gut microbiome, the community of microbes and associated genes residing in the gastrointestinal tract, has been postulated to contribute to neurobiological and behavioral development in children[12]. Evidence primarily from animal studies supports communication between the microbiome and the central nervous system, with effects on social, explorative, and affective behavior. This is mediated through several mechanisms, including the coordination between the neuroendocrine and immune systems, stimulation of the vagus nerve, and metabolism of neurotransmitters[13]. A human intervention trial manipulated urban environmental biodiversity in children to evaluate its effects on the commensal microbiome and immunoregulation; the study found that biodiversity intervention enhanced immunoregulatory pathways, manifested in increased plasma TGF-β1 levels, proportion of regulatory T cells, and plasma IL-10:IL-17 A ratio[14]. An environment set up to allow children to freely interact with nature comfortably could positively impact the influence of the microbiome on childhood outcomes. However, medical data substantiating this hypothesis are scarce[13,14].

Given the above, reducing gastrointestinal symptoms is vital for children's physical and mental health. However, no studies have linked these factors in young children. We hypothesized that childcare provided in a biodiverse natural environment could improve the health of children in early childhood with potentially long-lasting effects. This study aimed to investigate the effects of various levels of exposure to a rich natural environment (once weekly for one month) on gastrointestinal symptoms, salivary cortisol, salivary amylase, and gut microbiota. We verified the hypothesis that exposure to a rich natural environment reduces gastrointestinal symptoms in young children. Furthermore, we tested a secondary hypothesis that exposure to a rich natural environment reduces salivary cortisol and salivary amylase levels and a third hypothesis that this exposure increases the diversity of the gut microbiota.

Methods

Study design

This randomized trial was conducted in four kindergartens in Tohoku/Hokkaido, Japan. This study was approved by the Ethical Review Board of Tohoku University School of Medicine (approval number 2022-1-489) and registered with the University Hospital Medical Information Network (study number UMIN000047462, date of first trial registration: 11/04/2022). All experiments were performed in accordance with the Ethics Committee Tohoku University Graduate School of Medicine guidelines and regulations. Informed consent was obtained from the legal guardians of all study participants for inclusion in the study as well as for publication of their information/images in an online open access publication.

Participants

The inclusion criterion for this study was 5–6-year-old healthy children attending kindergarten. The exclusion criteria were a history of antibiotic treatment within the previous month, history of internal medicine or neurological diseases, and history of mental illness. After obtaining written informed consent from the children's parents and the participating children, the children were randomly assigned to one of two groups: a nature childcare group or a regular childcare group. Figure 1 shows a flowchart of the study. Of the 157 initially enrolled children, 27 did not provide consent; this was due to the children and their families either being too busy to take part or changing their mind regarding study participation. The remaining 130 children were assessed for eligibility and randomized. At the time the intervention trial was conducted, all children had been in childcare for between 29 and 30 months. All children received childcare from Monday to Friday for six hours per day, which was standardized because they all attended public kindergartens.

Randomization and masking

Cluster randomization was performed on a class-by-class basis within the kindergarten, and the children were with their class teachers and classmates to avoid unnecessary increases in stress. Randomization was performed by investigators not directly participating in the intervention trial using the RAND function in Microsoft Excel (Office 365, version 21.0; Microsoft Corporation, Redmond, WA, USA); those with values ≥ 0.5 were assigned to the intervention group, and the other classes inevitably became controls. These calculations were performed under strict control using a personal computer that others could not operate.

Procedures

This study employed waitlist control; in the regular childcare group, the participants were initially (for the first month) only exposed to the kindergarten environment, while in the nature childcare group, an additional period was allotted for a nature walk (Figs. 2 and 3). Children spent 4 h (between 09:00 and 13:00) in a natural environment. The natural environments provided as part of this study were grassland and forest landscapes located within a 5–10-minute walk from the children's kindergarten so as not to disrupt the children's kindergarten routine. Table 1 shows the timetable of activities that the children were exposed to in the regular and nature childcare groups. Further information regarding the intervention is provided in the supplementary material. The children were exposed to the respective conditions once a week for a month (a total of four times; Fig. 4). After one month, the children in the regular childcare group had the same experience as those in the nature childcare group. This was to keep the children's experiences fair, as requested by their teachers.

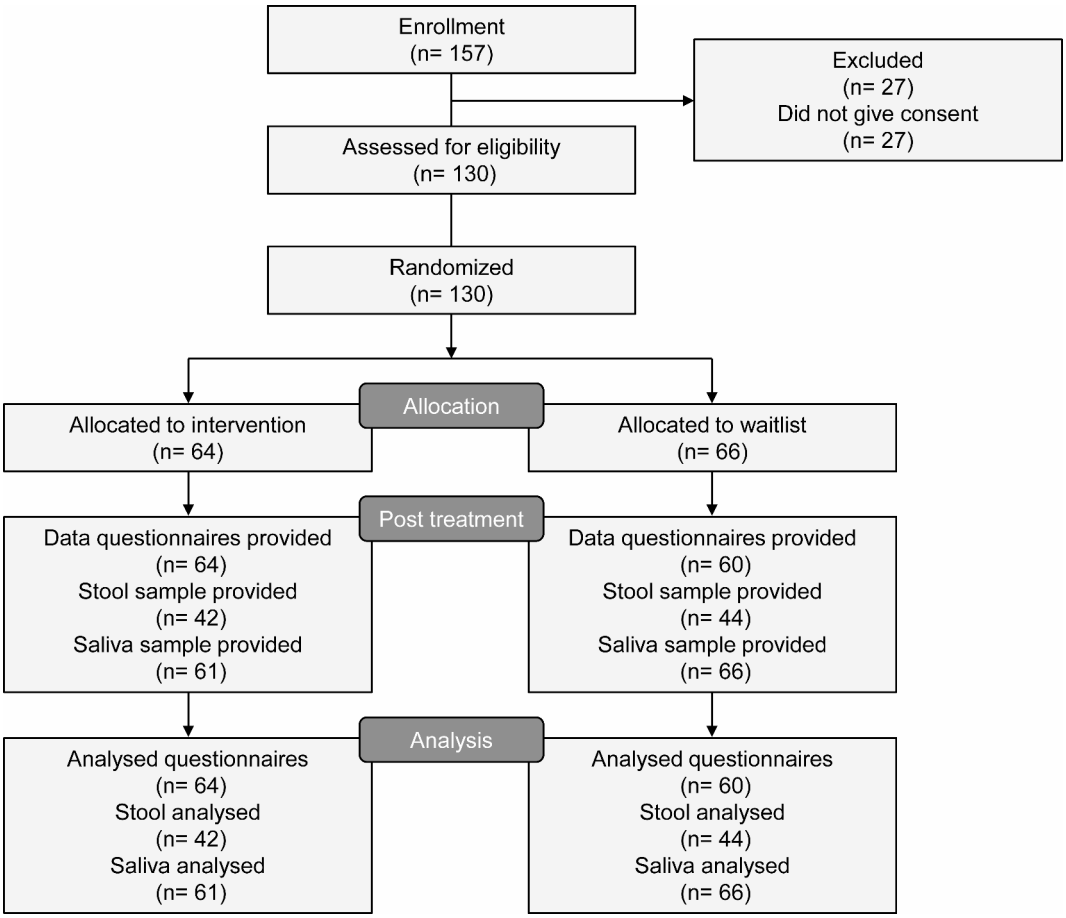

Fig. 1. Study flow chart.

The children engaged in activities along with trekking. These activities were similar to those provided during the daily childcare program. This was to prevent the activity from increasing curiosity or stress. Children from the four kindergartens had an equal opportunity to engage in all activities. The activities comprised the following:

- Crafts: Children made crafts using fallen leaves and branches.
- Science: Children used microscopes to examine objects they found in nature.
- The children read picture books and participated in a treasure hunt in nature.
- Painting: The children drew freely and worked on prints.

Outcomes

Before the intervention, gastrointestinal symptoms were detected using the Children's Somatization Inventory (CSI)[15]. The version of this questionnaire used in this study comprises 35 items that evaluate symptoms among children in the preceding two weeks; the 35-item version was utilized as it has been used in previous research in the field[3]. The following gastrointestinal symptoms of the questionnaire were grouped into a 'GI score': nausea and abdominal discomfort, abdominal pain, nausea after eating, bloating, constipation, diarrhea, and vomiting. If the GI score was ≥1, participants were categorized into the 'GI group,' and if the GI score was 0, they were classified as 'Controls.' The CSI has good reliability[16]. The CSI was administered and scored by the children's parents, who were not formally notified of their child's allocated group. Child behavior was analyzed using the Child Behavior Checklist for ages 4–18[17]. Fecal examination was performed for gut microbiota using 16 S-rRNA analysis, with the V1-V2 regions as targets (further information about the methodological details is provided below and in the supplementary material). Fecal analyses were performed by a professional vendor, resulting in a set of QIIME 2 bacterial flora analyses. Gut microbiota alpha diversity was analyzed using the Shannon index, calculated using QIIME 2 version 2020.8 (https://qiime2.org/)[18]. The Shannon index measures species diversity and evaluates the number of species and their relative abundance. Beta-unweighted and -weighted UniFrac analyses were also conducted using QIIME 2 to determine diversity metrics. The statistical tests performed are described in the 'Statistical analysis' section below. The salivary cortisol and salivary amylase levels were also measured using the methodology reported in previous studies[19,20]. Salivary samples were collected at 9 a.m. and 11 a.m. in both study groups before meals.

Data collected on the following factors after the intervention were the primary and secondary endpoints:

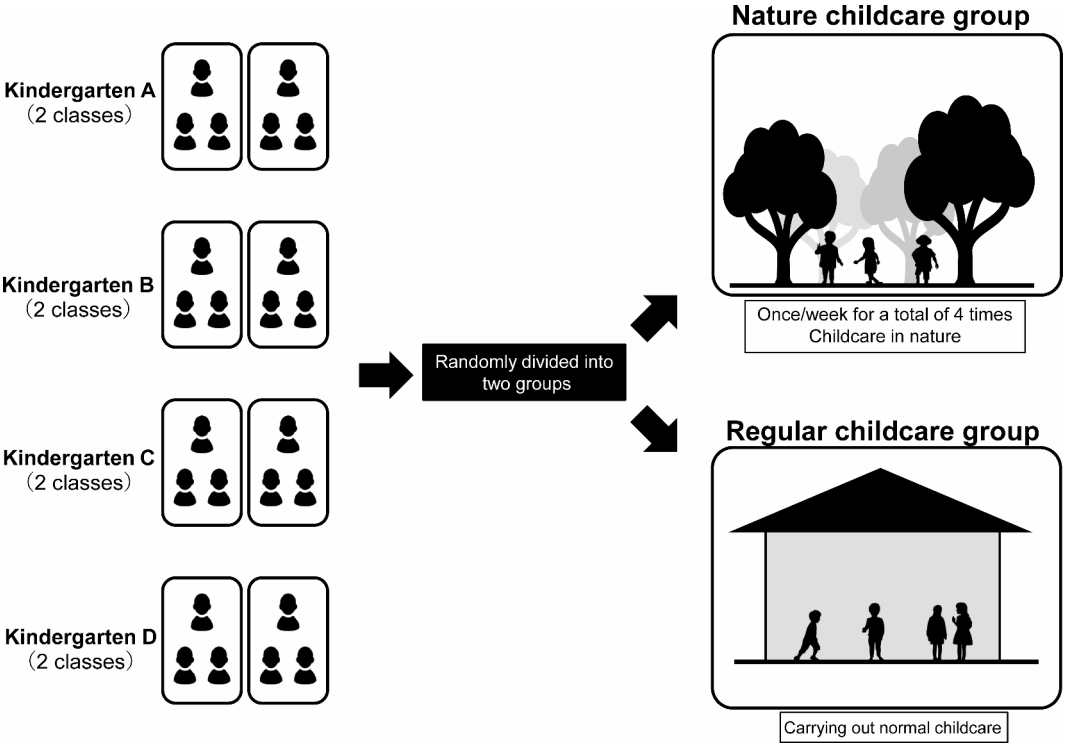

Fig. 2. Description of the study groups and the interventions.

(1) Primary endpoint: CSI GI scores.
(2) Secondary endpoint: gut microbiota (fecal examination).
(3) Secondary endpoint: salivary cortisol and amylase levels.
(4) Secondary endpoint: Child Behavior Checklist (CBCL 4–18 for PorT).

Fecal sampling, DNA extraction, and sequencing

Fecal samples were collected using Mykinso fecal collection kits containing a guanidine thiocyanate solution (Cykinso, Inc., Tokyo, Japan) and stored at 4℃ for one week. DNA was extracted from the fecal samples using an automated DNA extraction machine (GENE PREP STAR PI-1200 A, Kurabo Industries Ltd., Osaka, Japan) according to the manufacturer's protocol. The V1–V2 region of the 16 S rRNA gene was amplified using forward (16S_27Fmod: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGR GTT TGA TYM TGG CTC AG) and reverse (16S_338R: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTG CTG CCT CCC GTA GGA GT) primers using the KAPA HiFi HotStart ReadyMix (Roche). To sequence the 16 S amplicons on the Illumina MiSeq platform, we attached the dual-index adapters using the Nextera XT Index kit. Each library was diluted to 5 ng/μL, and equal volumes of the libraries were mixed to a concentration of 4 nM. The DNA concentration of the mixed libraries was quantified through qPCR using the KAPA SYBR FAST qPCR Master Mix (KK4601, KAPA Biosystems, Wilmington, MA, USA) with primers 1 (AAT GAT ACG GCG ACC ACC) and 2 (CAA GCA GAA GAC GGC ATA CGA). The library preparations were conducted according to the Illumina 16 S library preparation protocol (Illumina, San Diego, CA, USA). Libraries were sequenced using the MiSeq Reagent Kit v2 (500 cycles) and 250 bp paired-ends.

Taxonomic assignment based on 16 S rRNA gene sequences

The paired-end reads of the partial 16 S rRNA gene sequences were analyzed using QIIME 2 (version 2020.8)[5]. The steps for data processing and assignment based on the QIIME 2 pipeline were as follows: (1) DADA2 for joining paired-end reads, filtering, and denoising; and (2) assigning taxonomic information to each amplicon sequence variant using naive Bayes classifier in QIIME 2 classifier with the 16 S gene of V1-V2 region data of SILVA (version 138)[6] to determine the identity and composition of the bacterial genera.

Statistical analysis

The sample size was determined based on previous studies in the field and calculated using Power Analysis in SPSS version 29 (IBM Corp., Armonk, NY, USA) with the following parameters: significance level (α) = 0.05, power (β) = 0.8, and effect size (d) = 0.5 (moderate correlation). Sample sizes of at least 64 participants and 34 pairs were required based on this calculation for the two-group comparison and pre- and post-intervention comparison *t*-tests, respectively. The statistical analyses were performed using the same software. For normally distributed items, the *t*-test was used to compare the mean values, whereas the Mann–Whitney U test was used for non-normally distributed data. Chi-squared testing was performed to cross-tabulate sex, childcare, and

Fig. 3. Pictures illustrating the children in the nature intervention group (upper) and the regular childcare group (lower).

Time	Activity
9:00–9:20	Explanation of cautions in childcare details and childcare practice
9:20–9:30	Excretion confirmation
9:30–10:00	Free play (specify areas where the children can play considering their safety)
10:00–11:00	Tracking and walking
11:00–11:15	Excretion confirmation
11:15–12:00	Lunch (kindergarten-designated bento or school lunch)
12:00–12:35	Free play (specify areas where the children can play considering their safety)
12:35–13:00	Excretion confirmation, clean up, and board shuttle bus

Table 1. The daily schedule of participants.

maternal educational background. Alpha coefficients were calculated to assess the internal consistency of all 30 questionnaire items of the CSI and for the 7 GI score items alone. The Child Behavior Checklist scores were used to generate T-scores for participants for analysis. All participants were analyzed according to their originally assigned groups, and there was no crossover. Further analyses were focused on participants with confirmed gastrointestinal symptoms. Significant differences were verified with two-way ANOVA and post-hoc testing using the Bonferroni test. Mixed effects models were also created to facilitate differentiation between- and within-individual effects. P values < 0.05 were considered statistically significant.

For the microbiological statistical analyses, non-rarefied data were used, and the data were converted to relative abundance. Statistical analyses were performed using QIIME2 version 2020.8, with the package QIIME2

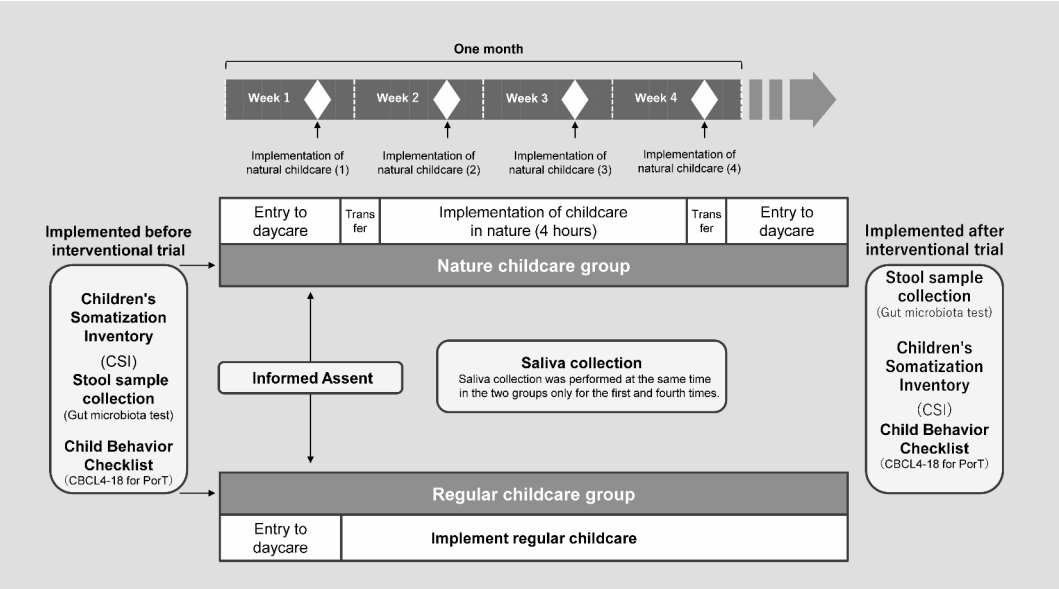

Fig. 4. Study protocol.

feature-table.relative-frequency used for the relative abundance conversion. For the beta diversity analyses, differences in Bray–Curtis distances between groups were assessed using a non-parametric permutation-based multivariate analysis of variation (PERMANOVA) test with 999 permutations[21] using the q2-diversity plugin in QIIME2, and principal coordinate analysis plots were generated. DADA2 was used for quality filtering, noise removal, and chimera removal. No samples had insufficient reads (number of 16 S rRNAs), and the final sample size was the total sample size of all subjects.

Results

Recruitment commenced on July 5, 2022, and finished on September 28, 2022. The follow-up period ended on November 30, 2022. Of the 130 participants randomized in this study, 64 were allocated to the nature childcare group (31 males and 33 females, mean age 5.59 ± 0.50 years) and 66 to the regular childcare group (32 males and 34 females, mean age 5.47 ± 0.50 years; Table 2). The groups were well matched at baseline, with no difference between the childcare groups in terms of sex and maternal educational background. Some randomized children did not provide completed questionnaires (parental refusal to submit the questionnaire), stool samples (constipation or child refusal), or saliva samples (absence during the saliva collection period; Fig. 1). Alpha coefficients for the total CSI items and just the 7 GI score items alone were both 0.776, confirming internal consistency. Before the intervention, the GI score derived from the CSI was non-significantly higher in the nature childcare group (2.48 ± 3.44) than in the regular childcare group (1.43 ± 2.32; Mann–Whitney U test result = 2131.0, Z = 1.104, effect size = 0.10, $p = 0.269$; Fig. 5). However, following the intervention, gastrointestinal symptoms in the nature childcare group were significantly lower than those in the regular childcare group (Mann–Whitney U test result = 1201.5, Z = − 4.136, effect size = 0.37, $p < 0.001$), particularly abdominal pain (Mann–Whitney U test result = 1565.5, Z = − 2.720, effect size = 0.24, $p = 0.007$) and constipation (Mann–Whitney U test result = 1692.0, Z = 0.146, effect size = 0.13, $p < 0.001$). Most symptoms in the nature childcare group decreased after the intervention, whereas no large change or clear pattern was observed in the symptoms among the regular childcare group (Supplementary Table S1). Supplementary Fig. S1 shows the changes in pre- and post-intervention scores at the individual participant level.

Table 3 presents the Shannon index data. No significant difference was observed in the overall before-and-after comparison or for each of the two groups. Furthermore, there was no difference in the Shannon index value between the nature and regular childcare groups before the intervention; however, the Shannon index value was higher in the nature childcare group than in the regular childcare group following the intervention (*t*-test value = 2.552, $p = 0.013$, effect size = 0.55, 95% confidence interval = − 0.347, − 0.043; Fig. 6). The beta-unweighted and -weighted UniFrac analyses (Supplementary Fig. S2), as well as the PERMANOVA testing (beta-unweighted group: pseudo-F = 0.983, $p = 0.514$; beta-weighted group: pseudo-F = 0.864, $p = 0.688$; Supplementary Fig. S3), confirmed no significant differences between the nature and regular childcare groups.

The participants' salivary cortisol and amylase levels are presented in Table 4; Fig. 7. Cortisol and amylase levels before the intervention were not significantly different between the two groups. However, after the intervention, salivary cortisol and amylase levels were significantly lower in the nature childcare group than in the regular childcare group.

To facilitate the differentiation of between- and within-individual effects in our longitudinal study, we performed mixed model analyses incorporating child-level variability and kindergarten as random effects, and childcare group (nature versus regular), time, and a childcare group × time interaction term as fixed effects. This analysis was performed to evaluate the effects of these variables on GI scores, salivary cortisol, salivary amylase,

	Nature group	Regular group	Total number of children in four centers
Total number of children	64	66	130
Boys	31	32	63
Girls	33	34	67
Age	5.59 ± 0.5	5.45 ± 0.5	5.53 ± 0.5
Psychiatric symptoms	2	1	3
Is the child in a special class at school?			
No	40	18	58
Yes	0	0	0
Has the child ever repeated a grade?			
No	38	18	56
Yes	0	0	0
Does the child have academic or other problems at school?			
No	36	18	54
Yes	0	0	0
Does the child have any kind of illness, physical disability, or intellectual disability?			
No	53	45	98
Yes	2	1	3
Playing outside: Average daily on weekday			
From waking time to 12 a.m.			
0 min	42	40	82
1–15 min	11	12	23
16–30 min	4	3	7
31–60 min	1	3	4
60 min or over	2	1	3
From 12 a.m. to 6 p.m.			
0 min	22	19	41
1–15 min	14	11	25
16–30 min	12	16	28
31–60 min	9	12	21
60 min or over	7	2	9
From 6 p.m. to bedtime			
0 min	50	51	101
1–15 min	5	3	8
16–30 min	2	3	5
31–60 min	2	2	4
60 min or over	1	0	1
Playing outside: Average daily on weekend			
From waking time to 12 a.m.			
0 min	10	9	19
1–15 min	12	4	16
16–30 min	10	15	25
31–60 min	17	23	40
60 min or over	11	8	19
From 12 a.m. to 6 p.m.			
0 min	3	4	7
1–15 min	5	7	12
16–30 min	12	15	27
31–60 min	26	15	41
60 min or over	18	20	38
From 6 p.m. to bedtime			
0 min	51	49	100
1–15 min	5	3	8
16–30 min	3	1	4
31–60 min	0	3	3
60 min or over	1	0	1
Continued			

	Nature group	Regular group	Total number of children in four centers
Playing outside: Average daily on weekday (min)	38.71 ± 58.01	33.68 ± 48.22	36.17 ± 53.13
Playing outside: Average daily on weekend (min)	96.12 ± 82.55	91.48 ± 68.57	93.74 ± 75.42
Average sleep duration (hour)	8.98 ± 1.40	8.99 ± 0.87	8.90 ± 1.04
Weekday sleep duration (hour)	8.83 ± 1.20	8.96 ± 0.87	8.90 ± 1.04
Weekend sleep duration (hour)	9.12 ± 1.28	9.10 ± 1.43	9.11 ± 1.35
Mother's educational background			
High school graduate or below	32	18	50
Diploma	13	15	28
Associate degree	6	9	15
Bachelor's Degree or higher	13	17	30
Sibling status			
Siblings present	52	49	101
Only child	12	10	22

Table 2. Demographic and baseline information of study participants.

and Shannon index values. Pairwise comparisons were conducted based on data obtained before and after the intervention (including the 9 a.m. saliva sample as the 'before' data and the 11 a.m. saliva sample as the 'after' data). These analyses, the results of which are shown in Supplementary Tables S2–S5, confirmed the lack of an effect of kindergarten on GI scores, salivary cortisol, salivary amylase, and Shannon index values. However, effects were seen for time ($F = 13.326$, $df = 122$, $p < 0.001$) and a childcare group × time interaction ($F = 12.890$, $df = 122$, $p < 0.001$) on GI scores of the childcare group ($F = 4.860$, $df = 122$, $p = 0.029$). Salivary cortisol values were affected by a childcare group × time interaction ($F = 20.744$, $df = 125$, $p < 0.001$). Salivary amylase values were affected by time ($F = 8.248$, $df = 125$, $p = 0.005$) and a childcare group × time interaction ($F = 13.991$, $df = 125$, $p < 0.001$). There were no significant fixed effects on the Shannon index. To ensure that other potential confounders did not influence the results, the mixed effects models were repeated with the addition of four covariates: maternal age, maternal educational background, and baseline time spent outdoors (the latter as two separate covariates: from waking time to 12 p.m., and between 12 p.m. and 6 p.m.). The results of this analysis identified the same significant variables as those identified in the models without covariates, with no new additional statistically significant results identified (data not shown).

Supplementary Table S6 presents the results of the Child Behavior Checklist. There were no significant differences in any element of the Child Behavior Checklist between the nature and regular childcare groups before and after the intervention. However, when comparing the results before and after the intervention within each childcare group (intragroup comparisons), significantly lower scores for the total score, introversion scale, extroversion scale, social withdrawal, and social issues were observed in the nature and regular childcare groups (Supplementary Table S6). A significantly lower attention issue score was noted following the intervention in the nature childcare group but not in the regular childcare group, whereas significantly lower misbehavior and aggressive behavior scores were noted in the regular childcare group but not in the nature childcare group. Supplementary Fig. S4 shows the changes in pre- and post-intervention Child Behavior Checklist scores at the individual participant level.

Mixed effects models were also developed for the Child Behavior Checklist scores, incorporating child-level variability and kindergarten as random effects, and childcare group (nature versus regular), time, and a childcare group × time interaction term as fixed effects. The results are summarized in Supplementary Tables S7–S17 and confirmed the lack of an effect of kindergarten on any of the measures of the Child Behavior Checklist. However, time showed an effect on Total Problems ($F = 35.069$, $df = 80.427$, $p < 0.001$), Internalizing Problems ($F = 19.416$, $df = 112.059$, $p < 0.001$), Externalizing Problems ($F = 25.013$, $df = 109.740$, $p < 0.001$), Depressed ($F = 15.574$, $df = 122.000$, $p < 0.001$), Anxious/Depressed ($F = 7.419$, $df = 115.910$, $p = 0.007$), Social Problems ($F = 15.331$, $df = 118.927$, $p < 0.001$), Attention Problems ($F = 6.803$, $df = 116.050$, $p = 0.010$), Rule-breaking Behavior ($F = 9.214$, $df = 118.838$, $p = 0.003$), and Aggressive Behavior ($F = 11.751$, $df = 111.505$, $p = 0.001$). There was also an effect of childcare group × time interaction on Somatic Complaints ($F = 5.653$, $df = 120.653$, $p = 0.019$).

Discussion

The current study aimed to investigate the effect of exposure to a rich natural environment on gastrointestinal symptoms, salivary cortisol, salivary amylase, and gut microbiota in young children, hypothesizing that exposure to a nature childcare intervention would reduce gastrointestinal symptoms, salivary cortisol, and salivary amylase, as well as increase the diversity of gut microbiota relative to those associated with exposure to regular childcare. We confirmed that most of these hypotheses were true by studying a group of approximately five-year-old children. However, an increase in gut microbiota diversity was only observed using a measure of alpha diversity (Shannon index) in the nature group following the intervention, and there were no significant changes in beta diversity. Another interesting finding was that no significant difference was observed when data from the entire cohort (including children with and without gastrointestinal symptoms) and data from only those with gastrointestinal symptoms were compared. This study's findings are noteworthy and emphasize the

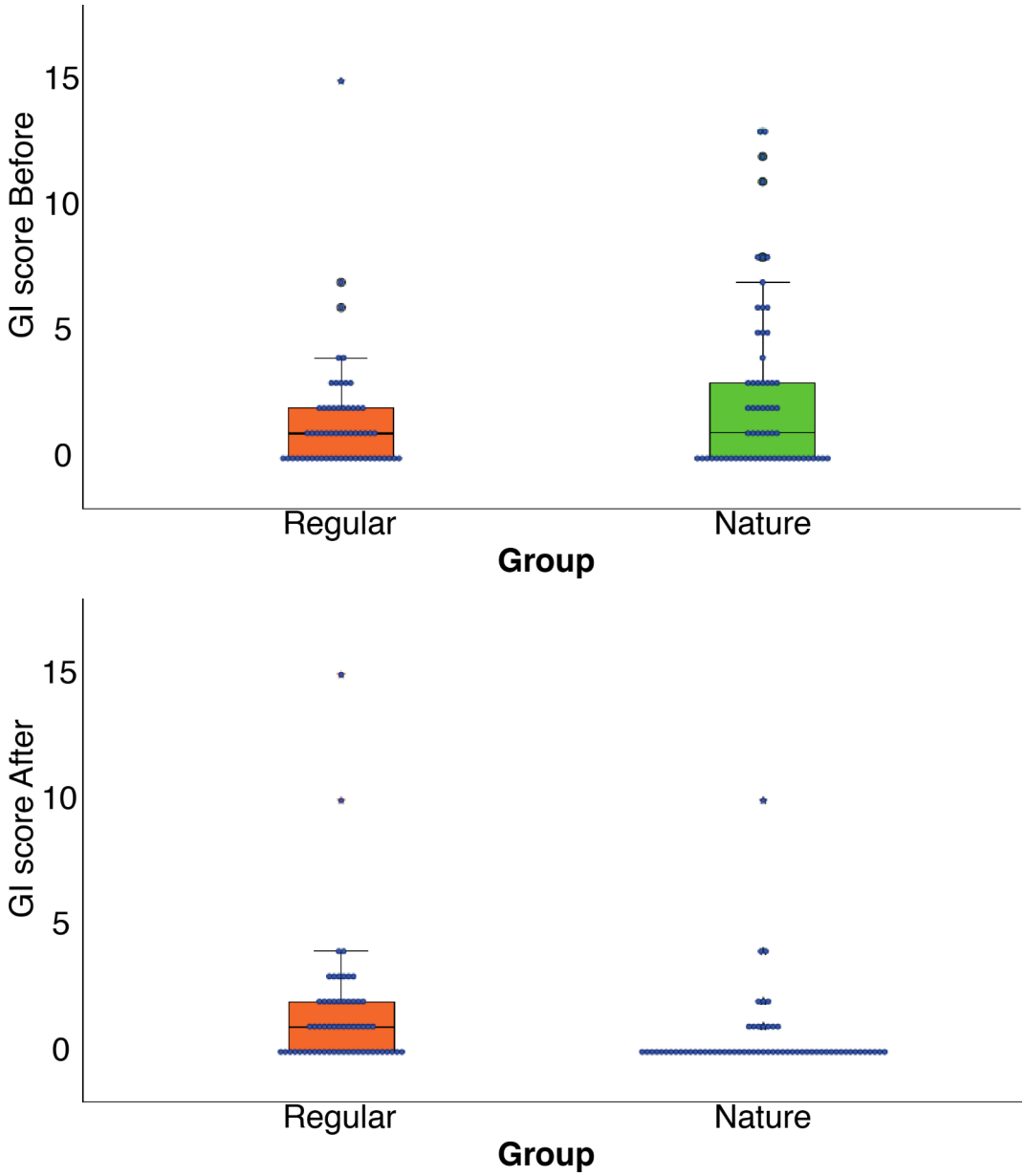

Fig. 5. Comparison of GI scores before and after the intervention in the two study groups.

Shannon index	Childcare group	*n*	Average	Standard deviation	Minimum value	Maximum value
Before intervention	Nature group	42	5.85	0.47	4.85	6.76
	Regular group	44	5.85	0.49	4.43	6.71
After intervention	Nature group	42	6.03	0.33	5.26	6.74
	Regular group	44	5.84	0.38	4.99	6.74

Table 3. Shannon index values by childcare group.

need to optimize children's long-term outcomes and potential. Given the improvements in gastroenterological symptoms and the biochemical findings, these data provide promising evidence of an approach to improve childhood psychosocial outcomes.

Children in the nature childcare group had fewer gastrointestinal symptoms than those in the regular childcare group (the primary endpoint). The proportion of children with gastrointestinal symptoms in our study was 55.56% in the nature group and 58.33% in the regular group, confirming the high prevalence of gastrointestinal symptoms in children, consistent with the 58% prevalence rate reported in a previous study of seven-year-old

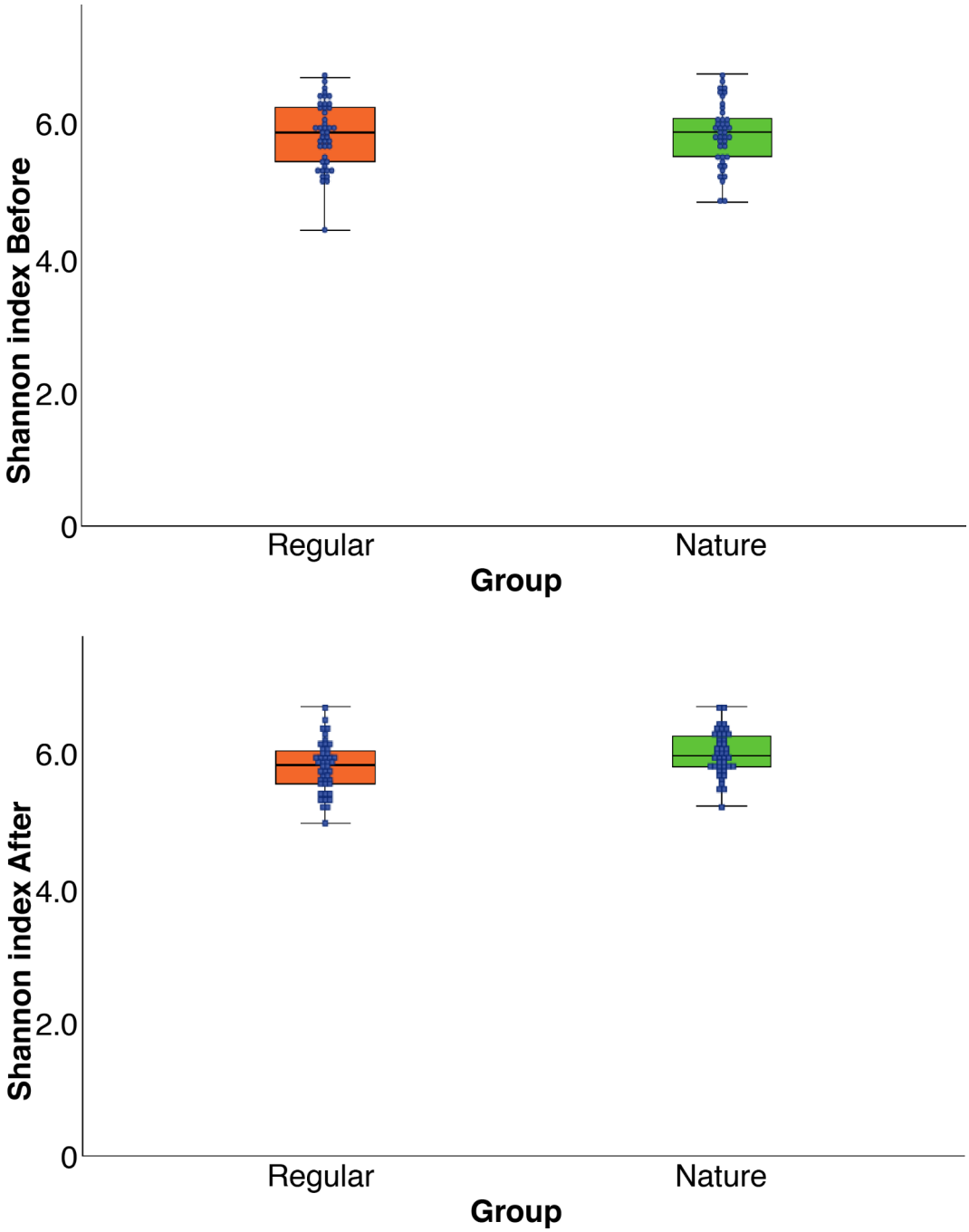

Fig. 6. Comparison of Shannon index scores before and after the intervention in the two study groups.

children[3]. Disorders of gut-brain interaction (DGBI, formerly known as functional gastrointestinal disorder) in children and adolescents profoundly impact children's health[22]. Gastrointestinal symptoms (abdominal pain, constipation, diarrhea, or psychosocial distress) in a child reflect which components of the brain-gut axis are affected and to what extent. Some children with IBS have rectal hyperalgesia[23]. Visceral hypersensitivity may be related to psychological distress (anxiety, depression, impulsiveness, anger)[24], with elevated salivary cortisol or amylase levels. Increased mucosal proinflammatory cytokines have been demonstrated in this context and may be induced due to acute infectious gastroenteritis (postinfectious IBS)[22]. Alterations in the gut microbiome have been demonstrated in pediatric patients with IBS[25,26]. Although this study was not limited to children with DGBI, the nature of the childcare intervention may have had a positive effect on gut health in children, although the beta diversity indices in the current study did not provide objective evidence of a change in microbiota driving the observed changes. Nevertheless, the other findings of this study warrant further investigation into the factors driving the change in GI scores observed.

	Childcare group	*n*	Minimum value	25th percentile	Median	75th percentile	Maximum value
Salivary cortisol levels before intervention	Nature group	61	1.00	1.20	1.70	2.40	7.90
	Regular group	66	1.00	1.20	1.50	1.90	8.20
Salivary cortisol levels after intervention	Nature group	61	1.00	1.00	1.40	1.90	5.30
	Regular group	66	1.00	1.60	2.35	3.30	8.50
Salivary amylase levels before intervention	Nature group	61	53.90	136.80	192.80	374.20	800.10
	Regular group	66	56.10	124.00	184.95	297.90	800.00
Salivary amylase levels after intervention	Nature group	61	29.40	131.10	191.50	382.00	800.00
	Regular group	66	5.60	175.20	287.70	507.80	800.00

Table 4. Cortisol and amylase levels by childcare group.

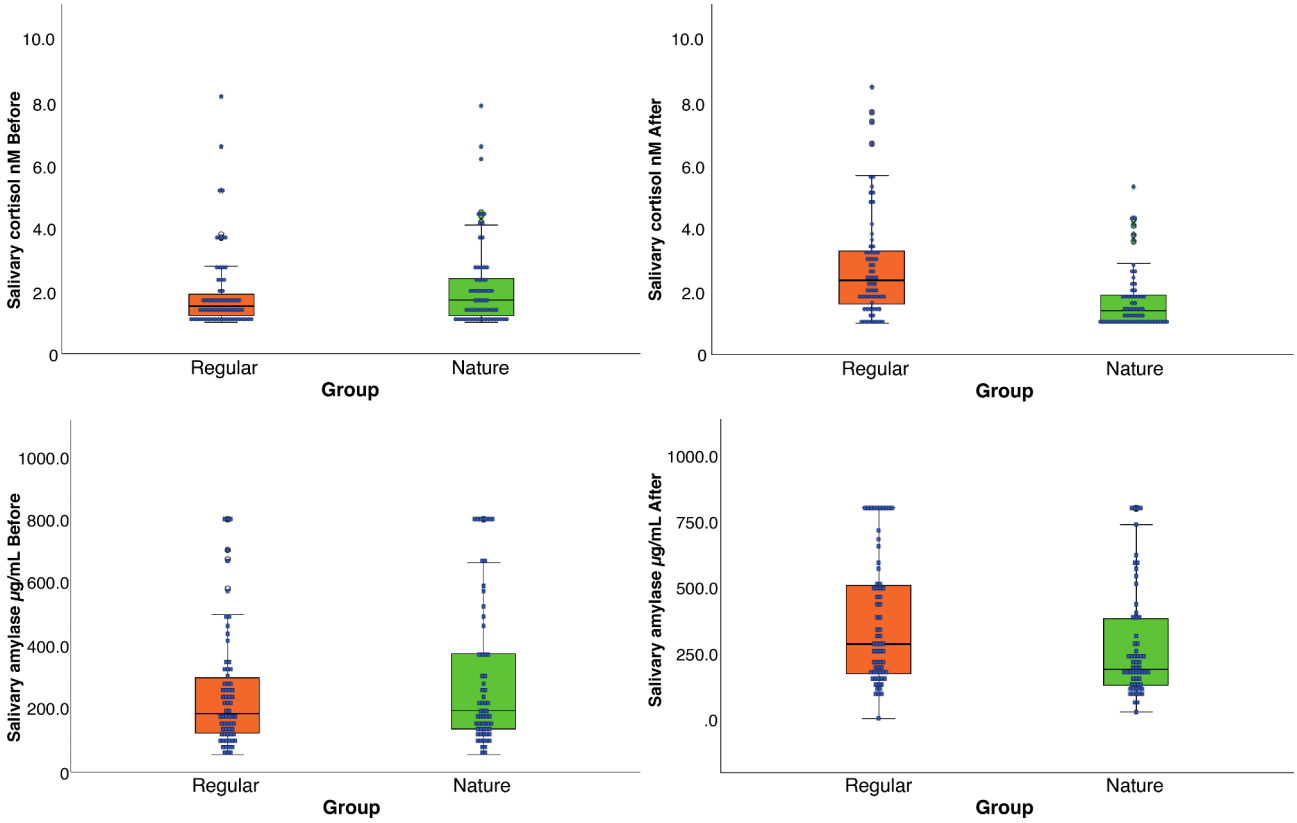

Fig. 7. Levels of salivary cortisol (top) and amylase (bottom) by childcare group before and after the intervention.

This study also provides evidence indicating a reduction in the levels of the stress hormone cortisol and amylase associated with care taking place in nature. Patients with IBS are characterized by increased levels of adrenocorticotropic hormone and cortisol secretion following the administration of corticotrophin-releasing hormone[27]. The baseline salivary cortisol level is increased in patients with IBS compared to that in controls, and the cortisol awakening response is blunted[7]. Changing gut microbiota in germ-free mice greatly influences adrenocorticotropic hormone and cortisol secretion to reduce stress[28]. Therefore, although relaxed mood and altered gut microbiota in the natural environment could be hypothesized to have accounted for the reduced salivary cortisol in the nature childcare group, the lack of strong evidence of altered gut microbiota in the current study challenges this. Changes in salivary amylase are also a marker of stress response[8]. Concordant changes in cortisol and amylase levels support the reliability of the stress reduction identified in the nature childcare group in the present study.

This study is unique but builds on previous studies that indicated a link between the gut microbiome and child development[12–14]. A previous interventional study on urban environmental biodiversity in Finnish children focused on the microbiome and immunoregulatory pathways[14]. The intervention diversified both the environmental and skin gammaproteobacterial communities, which, in turn, were associated with increases in plasma TGF-β1 levels and the proportion of regulatory T cells. They also found that children with naturally diverse environments had a more diverse gut microbiota[14]. However, the study was not a randomized controlled trial

like the current study. Furthermore, the current study expanded the parameters to incorporate gastrointestinal symptoms, gut microbiota, and biomarkers of stress response (salivary cortisol and amylase).

The composition of the human gut microbiota is linked to health and disease[29]. The diversity of gut microbiota is decreased in pathological conditions, especially in IBS[30]. Therefore, our findings of increased diversity in gut microbiota in the nature childcare group after the intervention and the decreased GI scores are meaningful. A previous study demonstrated that nature childcare centers showed more diversity in skin Proteobacteria alpha-diversity than standard childcare centers[14]. The presence of fecal *Faecalibacterium prausnitzii* was negatively associated with proinflammatory cytokine IL-17 A levels in the nature-oriented group only. It was suggested that the environmental microbiota may have adhered to the clothes, hair, face, lips, skin, hands, and fingers of the children in the nature care group. They can be ingested in the gastrointestinal tract via the oral route, resulting in an increased diversity of the gut microbiota.

Scores on many components of the Child Behavior Checklist, including the total score, decreased after the intervention in the nature and regular childcare groups, with no significant difference between the two groups. However, the results reflect the characteristics of the two groups. In normal childcare, scrambles and fights over playground equipment often occur, and the teacher usually corrects the child's aggression or misbehavior in such instances. One month of these instructions and corrections may have influenced the Child Behavior Checklist scores. However, children in the nature childcare group spent more time in nature than in a regular nursery. Nature is a vast environment; therefore, fights for playground equipment are unlikely. Children require attention in a natural environment because they can encounter small animals and flowers and need to be aware of dangerous terrains, insects, and flowers. These differences in the experiences of the children in the nature and regular childcare groups may explain the study's results.

This study had several limitations. First, although parents were not formally notified about which group their child was allocated to, they may have discovered this through discussions with their children. Thus, the outcome assessment of the CSI scoring may not have been truly blinded. However, this limitation would not have influenced the analysis of gut microbiota and salivary data, as this was not conducted by the parents. A second limitation was the inability to control the home environment, which is unavoidable in intervention trials such as the current study. Third, the current study did not provide long-term follow-up data to confirm the trends in GI symptoms, gut microbiota, and salivary amylase and cortisol levels. Fourth, data on laxative use were not collected as it is uncommon to use these medications in very young children such as those in the current study. Fifth, we did not collect data from participants on delivery method relating to their birth, the use of other medications, or pet ownership; this should be addressed in future studies. Finally, the difference in the time of saliva sample collection before (9 a.m.) and after (11 a.m.) the intervention could have influenced the results of the study; however, this was unavoidable due to the nature of the kindergarten curriculum. The strengths of this study include its randomized trial design and the collection of data from a range of outcome measures. In conclusion, our study supports the idea that spending time freely and abundantly in nature during early childhood could help maintain homeostasis of the digestive system and reduce the levels of the stress hormone cortisol. This may reduce the incidence of IBS. Further studies with larger populations and longer follow-up periods are required to confirm these findings and to explore the potential mechanisms driving these changes, particularly given the beta diversity results observed in this study.

Data availability

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

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Author contributions

C.S.: conceptualization, methodology, validation, investigation, writing – original draft; T.M.: formal analysis; S.S.: investigation; E.A.: formal analysis; S.W.: formal analysis; M.K.: validation; S.F.: supervision. All authors had full access to all data in the study and had the final responsibility for the decision to submit the manuscript for publication. C.S. and S.S. accessed and verified the data reported in this manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to C.S.