Childcare centre soil microbiomes are influenced by substrate type and surrounding vegetation condition

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HIGHLIGHTS

- Childcare centre soils are more bacterially diverse than sandpits.
- Soil bacterial communities are distinct between different soil substrates.
- Plant species richness and habitat condition influence soil bacterial communities.
- Soil areas in childcare centres may modulate child exposure to diverse bacteria.

ABSTRACT

Urban development has profoundly reduced human exposure to biodiverse environments, which is linked to a rise in human disease. The ‘biodiversity hypothesis’ proposes that contact with diverse microbial communities (microbiota) benefits human health, as exposure to microbial diversity promotes immune training and regulates immune function. Soils and sandpits in urban childcare centres may provide exposure to diverse microbiota that support immunoregulation at a critical developmental stage in a child’s life. However, the influence of outdoor substrate (i.e., sand vs. soil) and surrounding vegetation on these environmental microbiota in urban childcare centres remains poorly understood. Here, we used 16S rRNA amplicon sequencing to examine the variation in bacterial communities in sandpits and soils across 22 childcare centres in Adelaide, Australia, plus the impact of plant species richness and habitat condition on these bacterial communities. We show that sandpits had distinct bacterial communities and lower alpha diversity than soils. In addition, we found that plant species richness in the centres’ yards and habitat condition surrounding the centres influenced the bacterial communities in soils but not sandpits. These results demonstrate that the diversity and composition of childcare centre sandpit and soil bacterial communities are shaped by substrate type, and that the soils are also shaped by the vegetation within

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1. Introduction

Urbanisation is rapidly increasing across the globe and is a leading cause of biodiversity loss (Aronson et al., 2014; Flies et al., 2020; von Hertzen et al., 2011). It is projected that by 2050, around 70% of the global population will live in urban areas (UNDESA, 2019). This human population transition has reduced human engagement with naturally biodiverse environments, resulting in negative health impacts (Marseille et al., 2021; Robinson et al., 2022; Romanelli et al., 2015). One clear concept linking human health decline to increased urbanisation is the ‘biodiversity hypothesis’, which suggests that as urbanisation increases and biodiversity declines, our contact with diverse microbial communities (microbiota) is also reduced (Haahetla, 2019; Rook et al., 2003).

As we co-evolved in a bacterially diverse world, reduced contact with these ‘microbial old friends’ has likely impacted their roles in human immune system regulation (Haahetla, 2019; Rook et al., 2003). Direct evidence in support of the biodiversity hypothesis includes studies showing that children frequently in contact with biodiverse natural materials (e.g., moss, soil) have increased regulatory T cells and beneficial changes in cytokines compared to children without contact (Roslund et al., 2022). Moreover, rural children with higher exposure to diverse soil bacteria often have a lower risk of developing asthma (Stein et al., 2016), and contact with naturally diverse airborne bacterial communities from soil dust can modulate the gut microbiome in mice, with potential to reduce their anxiety-like behaviour (Liddicoat et al., 2020).

Further evidence of the biodiversity hypothesis comes from the high incidence rates of immune-related atopic disorders (e.g., asthma, hay fever) observed among populations of children living in urban areas (Ege et al., 2012; Roslund et al., 2021). The establishment and development of the gut microbiome and immune system occur primarily during early life stages and are influenced by exposure to a diverse range of environmental microbiota (Amir et al., 2022; Mulder et al., 2009). Insufficient exposure to such diverse beneficial microbiota (incl. bacteria, viruses, fungi and others) can lead to an inadequately trained immune system, immune dysregulation and heightened inflammation (Rook et al., 2015; Roslund et al., 2020; Rothchild et al., 2018). Initial colonisation of the gut with beneficial bacteria and other microbiota is facilitated through birth and breastfeeding (Martin and Sela, 2013), followed by dietary changes as infants grow (Tanaka and Nakayama, 2017). A child’s gut microbiome composition is also influenced by environmental factors such as pet ownership (Zhang et al., 2023), pollution exposure (Bailey et al., 2022), and the surrounding local environment (Tavalire et al., 2021). However, there is a substantial shift in the gut microbiome composition when children start childcare (Amir et al., 2022; Roslund et al., 2021). Indeed, childcare attendance can significantly influence the development of a child’s gut microbiome and, therefore, potentially promote the establishment and maintenance of a healthy immune system (Amir et al., 2022). Consequently, there is an opportunity to design and/or modify outdoor spaces in childcare centres to enhance children’s exposure to diverse environmental microbiota, thus supporting their health during critical developmental stages and helping to motivate the maintenance of biodiversity within childcare centres.

Young children spend a significant amount of time in childcare settings; for instance, a recent survey showed that 45% of children aged 0–5 years frequently attended Australian childcare centres (Australian Government, 2023). The outdoor play areas of childcare centres provide a space for children to potentially increase their exposure to beneficial microbial diversity via contact with common substrates, such as sandpits and soils (Robinson and Barrable, 2023). Indeed, Nurminen et al. (2018) showed that rubbing hands in bacterially rich soils led to increases in the diversity of skin and gut bacteria in adults. Hui et al. (2019) combined sand with a microbially rich inoculant and found an increase in skin bacterial diversity and reduced relative abundance of opportunistic pathogens in adults after contact. Furthermore, Roslund et al. (2022) conducted an intervention study whereby microbial inoculants from biodiverse soils were applied to sandpits that children played in. Exposure to these bacterially diverse sands resulted in shifts in blood markers toward enhanced immunoregulation in the children after contact. Other recent research (Robinson and Barrable, 2023) has recommended the inclusion of microbially-inoculated sandpits in childcare centres to support children’s contact with diverse bacteria, based on the findings by Hui et al. (2019) and Roslund et al. (2022). However, despite this research and the prevalence of sandpits and soils in Australian childcare centres, there remains a limited understanding of sandpit bacterial diversity and how it compares to that of different soil types commonly found in childcare centres (e.g., garden beds, lawns). There is also limited knowledge on how childcare management may influence sand and soil bacterial composition (e.g., sandpit sanitisation), as well as the influence of surrounding vegetation.

Plant diversity influences the diversity of bacteria in soil and air (Chalmandrier et al., 2019; Mhuireach et al., 2016; Robinson et al., 2021). Increased vegetation cover in residential areas affects both alpha and beta diversity in the microbiome (Styles et al., 2023), while plant species richness in urban areas is positively associated with soil bacterial diversity (Baruch et al., 2021). However, the relationship between plant diversity and bacterial communities in childcare centres remains unexplored. While increased plant species richness may affect soil bacterial composition in urban areas (Baruch et al., 2021), the impact of plant species richness on the bacterial communities of different substrates often found in childcare centres – e.g., sandpits, garden beds, lawns – is unknown. The habitat condition surrounding childcare centres may also impact soil bacterial communities. Indeed, surrounding vegetation has been previously shown to influence the microbiome and soil microorganisms in urban areas (Mhuireach et al., 2016; Robinson et al., 2021; Robinson et al., 2020). However, this relationship is yet to be studied in childcare centres.

Here, we sought to better understand how the diversity and composition of bacterial communities within childcare centre outdoor substrates is shaped by substrate type (i.e., sandpits, soils) and surrounding vegetation. To address these objectives, we asked the following research questions: (1) how does substrate (i.e., sandpit vs. soils) affect bacterial diversity and community composition in childcare centres in southern Adelaide? and (2) how do plant species richness and surrounding habitat condition influence the bacterial diversity and community composition in these substrates?

2. Materials and methods

2.1. Study sites and design overview

We recruited 22 childcare centres from across the Mitcham, Unley and Onkaparinga Councils in Adelaide, South Australia. We visited childcare centres over five weeks, from July 18 to August 23, 2022, to collect sand and soil samples for bacterial community and physicochemical analysis (described below). We also surveyed plant species richness and plant structural diversity at each childcare centre over a two-week period from December 2022 to January 2023. Additionally, we used modelled spatial data (derived from remote sensing) extracted from the LOOC-B online tool (https://looc-b.farm/) to quantify the habitat condition surrounding each childcare centre (described below).
2.2. Sampling sand and soil substrates

2.2.1. Bacterial community samples

We collected samples for bacterial 16S rRNA analysis from two substrates within each centre: sand from sandpits, and soil from yards. Yard soil was organised into four categories based on current centre management, as follows: soil from (1) lawns, (2) bark chip areas, (3) garden beds and (4) mud play areas (Table S1; note, not all centres had each of these categories; Fig. 1). A maximum of two samples of each substrate type was taken from each centre (e.g., if a childcare centre had two sandpits, we collected sand from both; see Table S1 for a breakdown of sample sizes by centre). For each sandpit/soil area, we collected five sub-samples of 120 g each from random points across the area to a depth of 10 cm using a decontaminated trowel (Cando-Dumancela et al., 2023; decontaminated with Decon 90 and ethanol, following protocols outlined in Cando-Dumancela et al., 2021). Sub-samples were pooled and homogenised in a sterile plastic bag (total ~600 g sand or soil). From each pooled and homogenised sample, we filled a 50 mL Falcon tube for DNA extraction, stored on ice in the field and placed in a 20 °C freezer within 5 h of collection. For each sandpit/soil area, we collected five sub-samples of 120 g each from random points across the area to a depth of 10 cm using a decontaminated trowel (Cando-Dumancela et al., 2023; decontaminated with Decon 90 and ethanol, following protocols outlined in Cando-Dumancela et al., 2021). Sub-samples were pooled and homogenised in a sterile plastic bag (total ~600 g sand or soil). From each pooled and homogenised sample, we filled a 50 mL Falcon tube for DNA extraction, stored on ice in the field and placed in a 20 °C freezer within 5 h of collection. We partitioned a further 250 g sample into a sterilised plastic bag and stored at room temperature for physicochemical analysis. Sandpits were categorised as sanitised or unsanitised based on information provided by childcare centre staff (sandpit sanitation is not currently required nor regulated in Australia https://www.acecqa.gov.au/); sanitation method and frequency varied across centres and included applying a commercial sandpit sanitiser and raking in salt.

2.2.2. Physicochemical samples

Samples were sent to CSBP Soil and Plant Analysis Lab (Bibra Lake, Western Australia) for analysis of the following properties: phosphorus, potassium, sulfur, organic carbon, nitrate nitrogen, ammonium nitrogen, electrical conductivity, pH (CaCl2), soil texture, and gravel % using CSBP protocols (described at https://www.csbplab.com.au/tests-soil). Where physicochemical values were lower than the minimum detection value, we used the minimum value for analysis (see Table S2 in Supplementary material).

2.2.3. DNA extraction, PCR and sequencing

DNA extractions from sand and soil samples were performed using Applied Biosystems MagMAXTM Microbiome Ultra Kit, following the manufacturer’s protocol (Thermo Fisher Scientific). Sample DNA concentrations were quantified using QuantiFluor ONE dsDNA System (Promega, Madison, WI, USA). The V3-V4 16S rRNA region of bacterial DNA was targeted using 341F/785R primers (Klindworth et al., 2013), and 1.5 ul of each DNA sample was PCR-amplified and sequenced using Illumina paired-end chemistry on the MiSeq platform at the Australian Genome Research Facility (AGRF; Adelaide, SA, Australia).

2.2.4. Bioinformatics

The QIIME2 bioinformatics pipeline was used to process raw reads to provide taxonomic classification and abundance of amplicon sequence variants (ASVs). Primers were removed from demultiplexed reads using the Cutadapt plugin (Martin, 2011). Figaro was used to determine the optimal trimming length for reads (Sasada et al., 2020), and then reads were trimmed and denoised using DADA2 (Callahan et al., 2016). An amplicon-region specific classifier was built using the q2-feature-classifier (Bokulich et al., 2018) and classify-sklearn naïve Bayes taxonomy classifier. ASVs were then identified using the SILVA database (version 138.1). We used R (R Core Team, 2022) to clean and filter ASVs before analysis. ASVs were removed if they were unidentified at the phylum level, classified as “Archaea”, “Mitochondria”, or “Chloroplast”, or were not found in at least two samples. Samples with low numbers of reads (<10,000 reads) were also removed (n = 5). The remaining samples (n = 85) were rarefied to the sample with the lowest read depth.

Fig. 1. Description and sample sizes of the five substrate types used in this study together with representative photos.
by specifying `sample.size (= 13,112 reads)` using the `rarefy_even_depth` function from the R `phyloseq` package (McMurdie and Holmes, 2013).

2.3. Vegetation data

2.3.1. On-site surveys

Plant species richness data were generated via a vegetation survey to identify the number of different plant species present in the childcare centres. The survey was conducted by systematically walking through each centre’s outdoor play areas to ensure all species were observed and recorded. A line was walked along one edge of the perimeter until the far side was reached while noting plants to the left and right along the line. Subsequently, a 2 m gap was crossed before continuing a new line down to the opposite end, and repeating to cover the entire outdoor area. This method was adapted depending on the shape and size of the childcare centre, which varied across centres.

2.3.2. Habitat condition

A measure of vegetation quality surrounding each centre was obtained via spatial data using the LOOC-B (Landscape Options and Opportunities Calculator – for Biodiversity) web-based calculator for ‘habitat condition’ (CSIRO, 2023). Habitat condition indicates the capacity of an area to support naturally occurring species in comparison to an ecological reference state and has a scale of 0–1. A value of ‘1’ suggests intact reference-like habitat that supports native biodiversity of flora and fauna, while ‘0’ indicates degraded areas with little capacity for providing habitat to naturally occurring species. This index is generated from two key sources: the National Forest and Scattered Woody annual layers (DISER, 2021) and the 90th percentile bare ground fractional cover annual layers (Geoscience Australia, 2021). The LOOC-B application programming interface tool was used to access and extract modelled habitat condition data at 100 m resolution for the geographic region containing childcare centres across southern Adelaide (Fig. 2).

Mean values were extracted for a radial buffer zone of 500 m surrounding each centre (for a more detailed description of the LOOC-B habitat condition model, see https://apidoc.looc-b.farm/overview.html).

2.4. Statistics

All statistics were performed in R studio with R version 4.2.2 (R Core Team, 2022). Soil bacterial community composition (beta diversity) was visualised using non-metric multidimensional scaling ordinations (NMDS) on Bray-Curtis distances, based on samples rarefied to 13,112 reads. We used a permutational multivariate analysis of variance (PERMANOVA) using the `adonis` function in the `vegan` package (Oksanen et al., 2012) to assess the effect of the predictor variables substrate type, sanitisation treatment, plant species richness, and habitat condition on bacterial community composition. A pairwise post-hoc test was run to identify differences in bacterial community between substrate types, using the ‘pairwise adonis2’ function. Ordination plots were annotated with ellipses (indicating groups) or trending directions only when significant compositional patterns were found. The homogeneity of dispersion was assessed using the `betadisper` function.

The alpha diversity of sand and soil samples was calculated using the exponential of Shannon’s diversity to obtain the effective number of ASVs (Jost, 2006). We used general linear models to explore the effects of substrate type, sanitisation method, plant species richness, and habitat condition on soil bacterial alpha diversity. Welch’s two-sample t-tests were used to determine differences in bacterial alpha diversity between substrate types and between sanitisation methods. A one-way ANOVA explored the difference in the effective number of ASVs between yard soil types. Simple linear regression with Pearson’s correlation coefficient was used to examine the relationship between bacterial alpha diversity and vegetation variables (plant richness and habitat condition), and the relationship was visualised on scatterplots.

Fig. 2. A map of southern Adelaide, South Australia, showing the childcare centre sampling region within the oval. Mean habitat condition values were calculated for a radial buffer zone of 500 m around each centre (not pictured here for sensitivity) using the CSIRO LOOC-B web-based tool (Background: OpenStreetMap; Datum: WGS84).
Assumptions of normality of residuals and homogeneity of variances were tested using Shapiro–Wilk and Levene’s tests, respectively, using the car package (Fox and Weisberg, 2019).

The geom_boxplot function in the ggplot2 package (Wickham, 2016) was used to visualise the differences in alpha bacterial diversity between substrate types, and between sanitisation treatments. The influence of plant species richness and habitat condition on alpha diversity were visualised using the functions geom_point and geom_smooth from the ggplot2 package (Wickham, 2016). Effective number of species data from all samples were log10 transformed, while effective number of species data from separate substrates were square root transformed to meet model assumptions.

The effect of substrate type on physicochemical characteristics was assessed using Kruskal–Wallis tests (n = 80) as model assumptions were not met. Dunn’s post-hoc analysis with Bonferroni correction was used to determine significant differences in physicochemical characteristics between substrate types. The association between environmental variables (physicochemical, plant species richness, habitat condition) and bacterial beta diversity was visualised and analysed with a canonical correspondence analysis (CCA) using the ordi step function in the vegan package (Oksanen et al., 2018). Variables that were highly correlated (r > 0.75) were identified and removed using the findCorrelation function in caret (Kuhn, 2008), and variables selected by the model were tested with a permutated ANOVA (999 permutations). All the above statistical tests excluded outliers, which were identified using the R default boxplot function as any data points observed above or below 1.5 times above the interquartile range; datasets were adjusted accordingly.

3. Results

We analysed a total of 85 soil and sandpit samples across 22 childcare centres, including 40 sandpit samples (29 unsanitised, 11 sanitised) and 45 soil samples across four categories (13 bark chip, 14 garden bed, 11 lawn, and seven mud play areas; see Table S1 in Supplementary material for a detailed breakdown of samples). Plant species richness across the 22 childcare centres ranged from 17 species to 162 species, with a mean of 60.63 ± 34.19 SD different plant species. Centre outdoor areas were dominated by herbaceous plants, followed by shrubs, trees, graminoids and succulents. Habitat condition surrounding each centre ranged from values of 0.002 to 0.164, with a mean of 0.043 ± 0.054 SD (see Table S3 in Supplementary material for a detailed breakdown of vegetation and habitat data).

3.1. Bacterial diversity and community composition

3.1.1. Effect of substrate type on bacterial alpha and beta diversity

After sequence data were cleaned and filtered, there were 2,536,411 total reads ranging from 13,112 to 103,688 reads per sample, with a total of 65,549 bacterial ASVs across the 85 samples (see Table S4 in Supplementary material for a detailed breakdown of samples).

Soils had greater alpha diversity than sandpits (t = −4.58, df < 0.001; Fig. 3a; soils: mean 459.72 ± 138.35 SD effective number of ASVs, sandpits: mean 325.76 ± 218.54 SD effective number of ASVs). Sandpit and soil samples also had distinct bacterial communities (PERMANOVA; df = 1, F = 6.714, R² = 0.075, p < 0.001; Fig. 3b). Beta dispersion was also significantly different (df = 1, F = 6.93, p = 0.014), with soil samples being more dispersed than sandpit samples.

3.1.2. Effect of soil type on bacterial alpha and beta diversity

The alpha diversity of soil bacteria did not differ between outdoor soil types (df = 3, F = 0.328, p = 0.805; Fig. 4a). However, bacterial community composition did differ between soil types (PERMANOVA; df = 3, F = 1.54, R² = 0.101, p < 0.001; Beta dispersion; df = 3, F = 1.99, p = 0.098; Fig. 4b). Specifically, differences were found in the bacterial communities between lawns and garden beds (df = 1, F = 2.0585, R² = 0.08215, p = 0.001), barkchip areas and garden beds (df = 1, F = 1.5174, R² = 0.05722, p = 0.003), lawns and mudplay areas (df = 1, F = 1.8496, R² = 0.10362, p = 0.005), mudplay areas and garden beds (df = 1, F = 1.4937, R² = 0.07289, p = 0.011), and between barkchip areas and lawns (df = 1, F = 1.3754, R² = 0.05884, p = 0.031). There was no significant difference between the bacterial communities of mudplay and barkchip areas (df = 1, F = 0.9768, R² = 0.05147, p = 0.518). The bacterial communities of garden beds were tightly clustered, likewise lawn bacterial communities were also clustered and distinct from other substrate types, while bark chip and mud play bacterial communities were more dispersed.

3.1.3. Effect of sanitisation treatment on sandpit bacterial communities

There was no effect of sandpit sanitisation on bacterial alpha diversity (unsanitised sandpits: mean 328.65 ± 221.16 SD effective number of ASVs, sanitised sandpits: mean 318.14 ± 221.86 SD effective number of ASVs; t = −0.133, df = 17.9, p = 0.895; Fig. 5a) or on beta diversity (PERMANOVA; df = 1, F = 1.068, R² = 0.027, p = 0.298; Beta dispersion; df = 1, F = 1.29, p = 0.26; Fig. 5b).

Fig. 3. (A) Boxplot of effective number of ASVs of sand compared to soil. (B) Non-metric multi-dimensional scaling (NMDS) plot using Bray-Curtis distances of bacterial community composition of different substrate types. Individual points represent samples, with colours denoting substrate.
3.2. Plant species richness and habitat condition

3.2.1. Effect of plant species richness and habitat condition on soil bacterial communities

Both plant species richness and habitat condition affected the community composition of soil bacteria (PERMANOVA; plant species richness: df = 1, F = 2.139, R^2 = 0.047, p < 0.01, habitat condition: df = 1, F = 1.55, R^2 = 0.035, p < 0.01; Fig. 6a, b). Beta dispersion was significant for plant species richness (df = 17, F = 5.42, p < 0.01) and habitat condition (df = 20, F = 4.41, p = 0.013). The alpha diversity of soils was not affected by plant species richness (F (1, 43) = 3.459, slope coefficient = -0.024, Pearson correlation coefficient = -0.273, p = 0.069; Fig. 6c) nor habitat condition (F (1, 43) = 1.155, slope coefficient = 8.408, Pearson correlation = 0.162, p = 0.289; Fig. 6d).

3.2.2. Effect of plant species richness and habitat condition on sand bacterial communities

Plant species richness and habitat condition had no effect on the bacterial communities found in sandpits (PERMANOVA; plant species richness: df = 1, F = 1.097, R^2 = 0.028, p = 0.27, habitat condition: df = 1, F = 1.321, R^2 = 0.034, p = 0.055; Fig. 7a, b). Beta dispersion was significant for plant species richness (df = 18, F = 12.89, p = 0.001). Plant species richness (F (1, 38) = 0.097, slope coefficient = 0.008, Pearson correlation = 0.05, p = 0.757; Fig. 7c) and habitat condition (F (1, 38) = 2.402, slope coefficient = 25.292, Pearson correlation = 0.244, p = 0.129; Fig. 7d) also had no effect on alpha diversity in sandpits.

3.3. Physicochemical characteristics

Substrate type strongly affected physicochemical characteristics. Sandpits consistently contained lower levels of most tested physicochemical properties compared to soils (ammonium H = 46.761 df = 4, p
< 0.0001; nitrate $H = 50.07$, df = 4, $p < 0.0001$; phosphorus $H = 55.269$, df = 4, $p < 0.0001$; sulfur $H = 38.668$, df = 4, $p < 0.0001$; organic carbon $H = 56.099$, df = 4, $p < 0.0001$; conductivity $H = 55.452$, df = 4, $p < 0.0001$; texture $H = 55.32$, df = 4, $p < 0.0001$; see Table S2 for details). The greatest difference in physicochemical characteristics was found between garden beds and sandpits (Bonferroni adjusted $p$-values $= p < 0.0001$). No difference was observed between the pH level of sandpits compared to soils ($H = 2.62$, df = 4, $p = 0.622$).

Overall, lawns had the highest levels of potassium, nitrate nitrogen, conductivity, and texture (potassium: mean $303.9 (\pm 173.10)$ SD mg/kg; nitrate: mean $127.60 (\pm 86.84)$ SD mg/kg; conductivity: mean $0.36 (\pm 0.16)$ SD dSm; texture: mean $1.84 (\pm 0.24)$ SD); see Table S4 in Supplementary material). Garden beds were highest in phosphorus, sulfur, and organic carbon (phosphorus mean: $116.43 (\pm 75.15)$ SD mg/kg; sulfur: mean $40.02 (\pm 39.76)$ SD mg/kg; organic carbon: mean $2.57 (\pm 1.15)$ SD mg/kg). Bark chip areas were highest in ammonium nitrogen (mean $3.91 (\pm 3.56)$ SD mg/kg), and sandpits had the highest pH levels (pH: mean $7.03 (\pm 0.60)$ SD; see Fig. S2, Table S4 in Supplementary material). Ammonium nitrogen, organic carbon, potassium, and pH variables best explained the variation in bacterial community composition, as summarised through CCA Axis 1 (Fig. 8).

### 4. Discussion

We investigated the bacterial diversity and composition of substrates (including sandpits and different soils) commonly found in childcare centre outdoor areas by sampling from 22 childcare centres across southern Adelaide, Australia. We characterised the bacterial communities via 16S rRNA amplicon sequencing and explored the effect of plant species richness and surrounding habitat condition on these bacterial communities. We show that sandpits had 41 % lower bacterial alpha diversity than soils, and that sandpit bacterial communities were distinct from soils. We also show that plant diversity within and habitat condition surrounding the childcare centres had a significant modulating effect on the composition of the soil bacterial communities. These findings highlight the importance of substrate choice and vegetation management in shaping the bacterial diversity and composition of common substrates found within outdoor play areas of childcare centres. Our study helps fill important knowledge gaps on the pathways of children’s exposure to potential health-promoting diverse environmental bacteria in childcare centres.

#### 4.1. Substrate type affects alpha and beta bacterial diversity

Our results suggest that when children have contact with soils in childcare settings, they are exposed to higher alpha diversity and different bacterial communities than when they have contact with sandpits. These results support the importance of children’s access to soils in outdoor play areas as they harbour greater bacterial diversity than sandpits, and a child’s contact with diverse bacterial communities has been experimentally shown to increased regulatory T cells and beneficial changes in cytokines compared to children without contact (Roshun et al., 2022). However, it is important to acknowledge that exposure to high bacterial alpha diversity does not necessarily always equate to favourable health outcomes. For instance, one sample could harbour more species than another, but the relative abundance of pathogens could also be higher. Therefore, additional research efforts
that include child health data plus those that distinguish pathogens from beneficial taxa while understanding the functional roles of microbiota in childcare settings are warranted.

Our finding that sandpits had lower bacterial alpha diversity than soils is consistent with studies that show that bacterial diversity is lower in sandy compared to silt and clay-based soils (Poll et al., 2003; Sessitsch et al., 2001), and that bacterial communities are shaped by different soil textures (Hemkemeyer et al., 2018; Xia et al., 2020). Sandpits are a common component of Australian childcare centres; therefore, it may be useful to further investigate the potential of solutions such as sandpit inoculation to increase the bacterial diversity of sandpits, especially considering that recent studies have found that sand inoculated with diverse bacteria may increase skin bacterial diversity (Hui et al., 2019) and modulate children’s gut and skin microbiomes with beneficial immune system effects (Roslund et al., 2022).

4.2. Soil type affects soil beta but not alpha bacterial diversity

We observed that bacterial community composition differed between soils from bark chip areas, garden beds, under lawns, and mud play areas. This suggests that children are likely to be exposed to different bacterial communities depending on which soil type they interact with. These results are consistent with previous works that show soil type can drive bacterial community composition; for example, differences in soil properties across a 1000 km transect of land in southeastern Australia were found to affect bacterial community structure (Lin et al., 2019; Xue et al., 2018). This information could be used to potentially shape childcare centre soil bacterial communities in a targeted way by changing their management (i.e., changing from lawn to mudplay areas). Future research could also investigate human health-associated bacteria (e.g., pathogens, commensal taxa) and other microbiota that may be found within these distinct communities.

We observed no significant difference in bacterial alpha diversity between soil types, as well as no significant difference in soil physicochemical results between soil types. Associations between soil physicochemical properties and bacterial alpha diversity have been demonstrated in previous work; for example, Han et al. (2022) recently showed that differences in soil chemical properties (e.g., increased nutrients via fertiliser) influenced bacterial alpha diversity across treatments in a tea plantation ecosystem. However, as we are not aware of any previous studies that have analysed bacterial alpha diversity across soil types in childcare settings, we recommend future studies build upon this preliminary study to detect potential differences in physicochemical properties and alpha diversity between soil types.

The health implications of this work rely on the transfer of these environmental bacteria into or onto children; however, investigating the specific exposure pathways or dose-response relationship between bacterial exposure via sand or soil substrates and children’s microbiomes was beyond the scope of this study. Previous work has shown that bacteria do transfer via the aerobiome pathway to the skin and respiratory tract through inhalation (Selway et al., 2020) plus onto skin and subsequently into the gut via dermal contact or ingestion via hand-mouth contact (Hui et al., 2019; Nurminen et al., 2018). Further experimental studies are required to deepen our understanding of these pathways, especially in childcare settings, for example through in situ exposure experiments alongside direct measures of child health outcomes, such as changes in skin bacterial diversity (Hui et al., 2019), gut microbiome (Nurminen et al., 2018), or immune markers in children (Roslund et al., 2022).

Fig. 7. Non-metric multi-dimensional scaling (NMDS) plot using Bray-Curtis distances showing the bacterial community composition of sandpits from centres with different (A) plant species richness and (B) habitat condition. Individual points represent samples with the colour range representing the level of plant species richness or habitat condition associated with centres; red represents high plant species richness or intact habitat condition and blue represents low plant species richness or degraded habitat condition. Scatterplots of the relationship between (C) plant species richness and effective number of ASVs, and (D) habitat condition and effective number of ASVs in sandpits.
4.3. Plant species richness and habitat condition influence soil beta diversity

The composition of soil bacterial communities in childcare centres was influenced by both the plant species richness within, and the habitat condition surrounding, each centre. These findings support the idea that surrounding vegetation influences soil bacterial communities (Baruch et al., 2021; Mhuireach et al., 2016; Robinson et al., 2021). Our results suggest that childcare centres with higher plant species richness in the outdoor play areas harbour different soil bacterial communities compared to centres with lower plant species richness. The condition of the surrounding habitat also appears to shape the bacterial communities found in childcare centre soils. These observed relationships between vegetation and soil bacterial communities are consistent with previous studies. For example, Baruch et al. (2021) showed that plant species richness positively affected bacterial diversity in soil from urban environments, while Chalmandrier et al. (2019) likewise found plant diversity to influence soil bacterial beta diversity. An important next step is to investigate the health-associated nature of the soil bacterial communities that may be influenced by higher vs. lower plant diversity and/or habitat condition. This could inform the development of vegetation management strategies for both within and surrounding centres, to shape specific soil bacterial communities to support children’s exposure to diverse bacteria.

Our study also found that although soil bacterial community composition was associated with both plant species richness and habitat condition, soil bacterial alpha diversity was not affected by these factors. Prober et al. (2015) previously showed plant diversity to be poorly related to the alpha diversity of soil bacterial communities but significantly associated with soil bacterial community composition, in line with our observation. However, Delgado-Baquerizo et al. (2018) reported contrasting findings over larger biogeographic scales, where soil biodiversity was linked to aboveground biodiversity and vegetation type. While our results may suggest that increased plant species richness would not affect soil bacterial alpha diversity, the robustness and interpretation of our results was potentially limited by the use of one measure of plant diversity (i.e., plant species richness), and the extraction of ‘habitat condition’ using the CSIRO LOOC-B website tool which has not yet been used in a research capacity (https://apidoc.looc-b.farm/overview.html). Therefore, future studies incorporating plant species abundance and plant phenotype data may deepen understanding of the important role that vegetation may have in shaping both the alpha and beta diversity of bacterial communities in childcare outdoor play areas.

4.4. Sanitisation has no observed effect on sandpit bacterial alpha or beta diversity

While we found no previous research examining the effect of sanitisation on bacterial diversity in sandpits, we expected to observe lower alpha diversity and altered beta diversity in sanitised sandpits compared to unsanitised. This expectation was based on the premise that sanitisation, by definition, eliminates microorganisms. However, here, no difference was observed in the alpha or beta diversity between sanitised...
and unsanitised sandpits. While this study was potentially underpowered, the results suggest that sanitisation may not effectively reduce bacterial diversity in childcare centre sandpits. Accordingly, further investigations are warranted that not only include a larger sample size but also collect more detailed information on sanitisation method. It is important to note that the baseline bacterial alpha diversity was already low in the centre sandpits, which is consistent with previous evidence that sandy substrates provide a poor habitat for bacterial communities (Poll et al., 2003). Our results show that bacterial community composition was not significantly affected by sanitisation. However, future studies could utilise different sequencing approaches (e.g., metagenomics, including mock communities) to explore the taxon present in sandpits in more detail, to discern the effect of sanitisation on the presence and abundance of mutualistic/commensal bacteria and opportunistic/pathogenic bacteria in sanitised versus unsanitised sandpits.

4.5. Conclusions

Our findings significantly advance our understanding of the microbial ecology of childcare centre outdoor spaces. The distinct microbial communities observed in sandpits and soils, with soils exhibiting greater diversity, suggest that these soils should be prioritised as a substrate in childcare centres to enhance children’s exposure to more varied microbial biodiversity. The impact of plant diversity within and habitat condition around centres on bacterial community composition implies that enhancing soil biodiversity could be achieved by augmenting on-site plant species diversity and improving the habitat condition in the vicinity of the centres. Future investigations into children’s exposure pathways to these environmental bacteria, such as aerobiome analysis and assessing children’s interactions and time spent with biodiverse substrates, will contribute crucial insights for the strategic design and modification of outdoor play areas to optimise children’s exposure to potentially health-promoting microbiota.

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Natalie S. Newman: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. Catherine A. Abbott: Writing – review & editing, Methodology, Conceptualization. Joel E. Brame: Writing – review & editing, Methodology, Formal analysis, Conceptualization. Christian Cando-Dumancela: Writing – review & editing, Resources, Investigation. Nicole W. Fickling: Writing – review & editing, Visualization, Data curation. Craig Liddicoat: Writing – review & editing, Visualization, Software, Formal analysis. Jake M. Robinson: Writing – review & editing, Visualization. Martin F. Breed: Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability


Data supporting ‘Childcare centre microbiomes are influenced by substrate type and surrounding vegetation condition’ (Original data) (Figgshare).

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Appendix A. Supplementary data

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