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Production, analysis, and safety assessment of a soil and plant-based natural material with microbiome- and immune-modulatory effects

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ABSTRACT

It has been suggested that reduced contact with microbiota from the natural environment contributes to the rising incidence of immune-mediated inflammatory disorders (IMIDs) in western, highly urbanized societies. In line with this, we have previously shown that exposure to environmental microbiota in the form of a blend comprising of soil and plant-based material (biodiversity blend; BDB) enhances the diversity of human commensal microbiota and promotes immunoregulation that may be associated with a reduced risk for IMIDs. To provide a framework for future preclinical studies and clinical trials, this study describes how the preparation of BDB was standardized, its microbial content analysed and safety assessments performed. Multiple batches of BDB were manufactured and microbial composition analysed using 16S rRNA gene sequencing. We observed a consistently high alpha diversity and relative abundance of bacteria normally found in soil and vegetation. We also found that inactivation of BDB by autoclaving effectively inactivates human and murine bacteria, viruses and parasites. Finally, we demonstrate that experimental mice prone to develop IMIDs (non-obese diabetic, NOD, mouse model) can be exposed to BDB without causing adverse effects on animal health and welfare. Our study provides insights into a potentially safe, sustainable, and cost-effective approach for simulating exposure to natural microbiota, which could have substantial impacts on health and socio-economic factors.

1. Introduction

The prevalence of autoimmune and inflammatory diseases, such as allergies, asthma and type 1 diabetes, has increased dramatically in recent decades, especially in western, highly urbanized countries [1,2]. Numerous studies have consistently revealed a direct relationship between atopic diseases, urbanization and a western lifestyle, and the protective effect of a farm environment and non-affluent traditional lifestyles against these diseases [3,4,5,6]. The biodiversity hypothesis proposes that exposure to the natural environment including vegetation and soil modulates the human microbiome, contributes to immune balance, and protects against allergy and inflammatory disorders [7]. This hypothesis is based in part on observations that the increase in the prevalence of immune mediated inflammatory diseases (IMIDs) correlates with a rapid loss of global biodiversity (reduction in biological species), which is most likely mediated by reduced contact with natural microbial biodiversity [8]. Indeed, it has been suggested that the disconnection of humans from soil (and the microbiota it harbors) in urban environments where soil is replaced by concrete and asphalt may be one of the reasons behind a compromised immune function and a high incidence of immune-mediated disorders in the western urban society [9]. This will likely be an even more serious issue in the future since it is believed that two-thirds of the world population will live in urban areas by 2050 [10].

Microbes and microbial components are proposed to modulate the immune system through continuous activation of pattern recognition receptors (PRRs) on both skin and mucosal surfaces [11]. This phenomenon is particularly abundant in rural natural environments and less

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so in artificial urban settings. Studies have shown that exposure to a highly diverse environmental microbiota and many microbial antigens, rather than a particular or selected microbial strains, is beneficial for immunoregulation and protection against immune-mediated disorders. Increasing evidence suggests that it is not only the diversity and richness of microbial exposure but also the specific types of bacteria, such as those found in soil, that are critical for the effective modulation of immune function [12,13,14]. In addition, exogenous microbiota may also alter the composition of commensal microbiota including those of the skin and gut in humans and animal models [15,16]. The human commensal microbiota, particularly the gut microbiota and their cellular components and metabolites, have a well-documented influence on the development and function of the immune system even though the specific roles of different microbes or the impact of their absence on immunological maturation are still poorly characterized, especially in humans [17,18]. In line with this, several studies have suggested that high exposure to soil microbes (and microbial components) and vegetation may be beneficial, and even necessary for the normal maturation of the immune system and protection against allergic and atopic diseases [19,20,21,22,23].

A few studies have explored exposure to soil and plant materials as a strategy to modulate the composition of commensal microbiota and immune function in animal models [16,24]. These studies have indicated that exposures can alter the composition of gut microbiota substantially and promote immune regulation. However, the exposures were carried out using one or very few soil or plant components. The microbial composition of soil depends on several environmental and anthropogenic factors [25] which may lead to problems with reproducibility if the collection time and area as well as storage conditions of the soil and plants differ [26]. In addition, a particular soil product, such as sand or a mixture of sand and peat, may comprise of a limited composition of microbes [27]. Consequently, it may neither stimulate an adequate number of PRRs, nor contain a sufficient number and richness of bacteria to produce immunomodulatory metabolites, resulting in limited immunomodulation. Likewise, the published studies [16,24] did not address the safety aspects of the exposure, which is of importance. Therefore, an extensive range of microbiota from a variety of natural sources that can reproducibly stimulate a broad range of PRRs and provide immune modulatory metabolites in a safe manner is desirable [28].

We have recently manufactured a biodiversity blend (here denoted BDB) from a variety of soil and plant-based materials as a potential immunomodulatory extract. The goal was to produce a blend with a high microbial biodiversity and density of non-culturable bacteria. In our previous studies we found that exposure to BDB altered skin and gut microbiota and immune status in both children and adults [29,30,31]. These findings suggested that BDB may mimic exposure to nature and that BDB could become a valuable tool to address the lack of such natural exposures more directly in the aetiology of inflammatory diseases. BDB may even be a promising candidate for the prevention of IMIDs. However, live BDB may pose a threat to immunocompromised people or experimental animal disease models since soil contains potential human and animal pathogens. Therefore, whether inactivation of live bacteria maintains the effect of BDB on microbial composition and the immune system in exposed individuals is of interest when considering BDB in preclinical and clinical trials, particularly if the intended routes of administration differ from topical application e.g. in an ingestible manner.

The composition of the BDB, its manufacturing process and microbial analyses have not been described in detail. In this report, we describe the composition and production of multiple BDB batches and how the production has been standardized. Additionally, we outline an effective method for inactivating potential human and murine pathogens in the BDB, ensuring its safe use in experimental animals and potentially immunocompromised humans. Finally, we address the safety of exposure to inactivated BDB in a mouse model known to develop IMIDs.

2. Material and methods

2.1. Material composition and manufacturing of BDB

BDB is a soil and plant-based natural material with powder-like constituency created by blending the raw materials and subsequent water-extraction, filtration and freeze-drying. The raw materials used in BDB are different forms of soil in varying proportions (referred to as major and minor components) and sphagnum moss. The major components consist of leaf compost, industrially manufactured agricultural soil and compost made of forestry and agricultural side streams in equal proportion (29.6 % each). These together constitute 89 % of the BDB. *Sphagnum* moss, the dominant moss of Nordic countries (https://artfakta.se/naturvard/taxon/1004718), was added to a final proportion of 7.4 %. The minor components added were potting soil, meadow soil, lawn soil, infrastructure/park soil and gardening soil at 0.7 % each (Table 1). The components of the BDB were chosen after careful consideration of microbial richness and diversity, ease in availability, and the presence and abundance of potentially pathogenic microorganisms. The difference between the major components and minor components was primarily the microbial diversity with the major components having a higher diversity compared to the minor components [26]. As previously reported, there were only minor variations in the microbial composition, dry weight, organic matter, pH, soil characterization, nutrients (NH_4^+, No_3^-, PO_4^-) and elements including Cd, Al, Co, Cr, Cu, Mn, Ni, Fe, Zn, P, As, and Pb before and during the storage time [26]. All raw materials were composted and controlled according to EU regulations [30].

The raw materials were stored in containers covered by lids that were perforated with two holes containing thin pieces of cotton to allow aeration but to prevent fast drying [26]. The containers were stored in a cold room (5 − 7 °C) until mixed thoroughly together. The major ingredients were pre-sieved through a Ø 5 mm sieve size and the minor ingredient using a Ø 2 mm sieve. Moss was dried overnight in a tray dryer and then crushed and mixed thoroughly before mixing with other ingredients. Ultra-pure sterile milli Q water was poured into the mixture little by little until the soil was saturated. The soil water mixture was incubated at room temperature for 3 h to allow for the extraction of microbes into the water phase as much as possible. The resulting extract was filtered through 125 μm sieves to collect the water extract and smaller particles. The size of the sieve was chosen to ensure homogeneity of the filtrate without a risk of clogging during the process. The flow through was collected in a new sterile bucket and transferred into

Table 1
Material composition of the biodiversity blend.

Material	Trade (Finnish) name	Percentage (volume)	Supplier
Major ingredients			
Leaf compost	Lehticomposti	29.6	Kekkilä, Finland
Agricultural soil	Maatalousmaa	29.6	Kekkilä, Finland
Forestry-agricultural side stream compost	Metsä-maatalouden sivuvirtakomposti	29.6	Biolan, Finland
Sphagnum moss	Sammal	7.4	Biolan, Finland
Minor ingredients			
Potting soil	Musta multa	0.7	Biolan, Finland
Meadow soil	Niittymulta	0.7	Kekkilä, Finland
Lawn soil	Nurmikkomulta	0.7	Kekkilä, Finland
Infrastructure/park soil	Inframulta	0.7	Kekkilä, Finland
Gardening soil	Viljelymulta	0.7	Kekkilä, Finland

sterile containers and frozen at −20 C overnight. The frozen material was then freeze dried at 0.25 bar pressure until the complete removal of moisture to finally obtain a powder-like formulation of BDB. The freeze-dried BDB was stored in sealed containers at 5–7 °C. To date, eleven batches have been produced using this method (with a few variations, as listed in Supplementary Table 1) and analysed in detail.

2.2. Microbial composition analysis of BDB

Total DNA was extracted from 0.25 ± 0.07 g of BDB in triplicates, which were then subsequently pooled, using PowerSoil® DNA Isolation Kit (MolBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's standard protocol. Sterile water (250 μl) was used as a negative control. The quality of the extracted DNA was checked using agarose gel (1.5 %) electrophoresis and quantified with Quant-iT™ PicoGreen® dsDNA reagent kit (Thermo Fisher scientific, MA, USA).

Sequencing of bacterial DNA from the BDB was done as described earlier [30]. Briefly, the V4 region within the 16S rRNA gene of bacterial DNA was amplified by PCR and sequenced using Illumina MiSeq platform (Illumina, San Diego, CA, USA). Raw sequences were processed following the Divisive amplicon denoising algorithm 2 (DADA2) pipeline (version 1.16) in the R environment (version 4.2.1) using the 'dada2′ package [32,33]. Quality profiles were generated for raw sequences, followed by trimming and filtering to remove low-quality bases, primers, and sequences with ambiguous bases using the 'filterAndTrim' function. Forward and reverse reads were merged using function 'mergePairs'. Dereplication was performed to reduce computational load by identifying and collapsing identical sequences. A sequence table was constructed and chimeric sequences were identified and removed using the 'removeChimeraDenovo' function. Dereplication and denoising were utilized using DADA2 default parameters. Taxonomic classification was achieved using the SILVA reference database (version 138) [34] with the 'assignTaxonomy' function. Sequences belonging to archaea, mitochondria and chloroplast, which accounted for 2.3 % of the total sequences (53230 out of 2,303,697 sequences), were removed. The alpha diversity (Shannon index and observed species) as well as the relative abundance of bacterial taxa were visualized using ggplot2 function in R (v 4.3.2).

Raw sequences generated in the study are available in Sequence Read Archive in NCBI under accession number PRJNA1037346.

2.3. Inactivation of BDB

BDB (around 50 ml) was transferred into individual autoclave bags under sterile conditions. Sealed bags were sterilized by autoclaving at 121 °C or 134 °C. Autoclaved BDB was stored at 4 °C prior to microbiological analyses and usage.

2.4. Human pathogen analyses

Total nucleic acid (DNA and RNA) was extracted from BDB by PowerSoil DNA Isolation Kit or PowerSoil Total RNA Isolation Kit (Qiagen, Hilden, Germany). qPCR was carried out for viral and protozoan pathogens including enterovirus, rhinovirus, rotavirus, norovirus, Giardia and Cryptosporidium as described in Krogvold et al. [35]. A cut-off Ct value of 42 was used to identify positive samples. The qPCR tests were validated by spiking samples with known amount of target RNA. Spiked samples were analysed by qPCR and results were compared to positive controls.

2.5. Rodent pathogen analyses

Genomic DNA was extracted from both autoclaved and non-autoclaved BDB using DNeasy Powersoil DNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer's standard instructions and quantified using nanodrop ND-1000 spectrophotometer (Thermo Fisher scientific, MA, USA). Total RNA was extracted from 50-100 mg BDB using TRIzol™ reagent (Thermo Fisher scientific, MA, USA) according to manufacturer's instructions and diluted in 50 μL sterile MilliQ water. The BDB was homogenized at 15 Hz speed with 1.25 mm beads for 8 min in a Tissuelyzer (Thermo Fisher scientific, MA, USA) in 1 mL TriZol prior to extraction. Extracted RNA was stored at −80°C until downstream analyses.

BDB (prior to or post autoclaving at 121 °C or 134 °C) was analysed for the presence of rodent pathogens according to the list of Federation of European Laboratory Animal Science Associations (FELASA) recommended quarterly check [36]. All testing was performed at a specific-pathogen-free (SPF) testing accredited facility (https://felasa.eu/) at the National Veterinary Institute of Sweden, Uppsala, Sweden. Bacterial outgrowth from aerobic and anaerobic cultures were analysed with MALDI-TOF at the species level while DNA or RNA extracted form live or autoclaved BDB was screened for viruses, parasites and bacteria by qRT-PCR (Table 2). The efficacy of pathogen inactivation was tested by autoclaving in standard (50 ml) or larger volumes of BDB and the reproducibility tested by autoclaving the BDB in three different runs.

2.6. Inactivated BDB exposures in mice

A breeding colony of non-obese diabetic (NOD) mice (strain NOD/Shi) were originally obtained from The Scripps Research Institute, La Jolla, USA. Animals were bred and housed in a specific pathogen free (SPF) environment at the Karolinska University Hospital, Huddinge. NOD mice are prone to develop autoimmune type 1 diabetes at an age above 10–12 weeks [37,38]. Diabetes incidence is high in female mice (around 60–90 % at an age of 6 months) but low in males (around 10–25 % at an age of 6 months). We initially exposed young nondiabetic male mice to the inactivated BDB over a three week period, and when the

Table 2
FELASA listed murine pathogens analysed by PCR, RT-PCR or culture-based methods.

FELASA listed pathogens	Detection method		
	PCR, RNA	**PCR, DNA**	**Culture**
Viruses			
Murine hepatitis virus	X		
Murine rotavirus	X		
Murine norovirus	X		
Murine parvoviruses		X	
Thelier's murine encephalomyelitis virus	X		
Bacteria			
Citrobacter rodentium			X
Clostridium piliforme			X
Corynebacterium kutscheri			X
Helicobacter spp. (*H. hepaticus*, *H. bilis* and *H. typholonius*)		X	
Mycoplasma pulmonis		X	
Rodentibacter heylii			X
Rodentibacter pneumotropicum			X
Salmonella spp.		X	
Staphylococcus aureus		X	
Streptobacillus moniliformis			X
Streptococci, beta haemolytic (not group D)			X
Streptococcus pneumoniae			X
Endoparasites			
Spironucleus muris		X	
Enterobius spp.		X	
Exoparasites			
Myobias spp.		X	
Rhadfordia spp.		X	
Myocopte spp.		X	

treatment proved safe, explored a longer exposure (6 weeks) in young non-diabetic female NOD mice. Mice in the exposure group were exposed to BDB autoclaved at 134 °C and the mice in the control group were mock exposed to the cage bedding material alone. Exposures were carried out in an SPF accredited facility and mice were allowed to acclimatize to the exposure room for a few days before the start of exposure. Exposure to BDB was carried out by sprinkling one pack of 50 mL autoclaved BDB over cage bedding material in a new cage. Mock exposures were always carried out before BDB exposures, and exposures were carried out at the same time of day throughout the study. Mice from a single cage (maximum of 5 in each cage) were transferred to the cage containing the BDB or bedding alone and were allowed to explore freely for 30 min after which they were transferred back to their original cage. A new (unused) cage was used for each exposure for the BDB and mock groups. The cages were opened and animals handled inside a laminar hood. The exposure dose chosen was based on our previous observation that this quantity of BDB caused a noticeable visual exposure effect such as presence of the BDB on body parts including fur, tail, nose, mouth, and ears [39]. Male NOD mice were exposed to BDB (n = 5) or mock (n = 5) five times a week (Monday to Friday) for three weeks and were left unexposed for a further six weeks to monitor the (long term) effect of BDB after exposure was discontinued. Female NOD mice were exposed to the BDB (n = 9) or mock (n = 8) five times a week (Monday to Friday) for six weeks. The animals were given free access to food and water throughout the experiment and were kept in the same room in an individually ventilated cage (IVC) system. Animal health was monitored by weekly weighing as well as through FELASA scoring that takes into account different parameters including the visual inspection of animal status and behavior (Supplementary information 1). Blood glucose levels were measured for all mice from tail vein blood at the experimental endpoint to establish whether any developed diabetes as described earlier [38].

Ethical approval for all animal work was obtained from Stockholm Southern Animal Ethics Board. Mice were housed and maintained in accordance with EU Directive 2010/63/EU for animal experiments.

2.6.1. Pulmonary health assessment

At experiment endpoint, mice were killed using isofluorane followed by cervical dislocation. Whole lung was fixed in 4 % PFA over-night, then transferred to 70 % ethanol until dehydration and embedding in paraffin. Single 5 μM sections were stained with hematoxylin and eosin and scored by a researcher blinded to the treatment groups. Overall health of the pulmonary structure was based on the level of perivascular and peribronchial inflammation, fibrosis, parenchymal and alveolar damage, and red blood cell infiltrate. Ordinal degree scores were set based on the total overall detection of unhealthy morphology (0–4; absent, rare, multifocal, coalescing, diffuse) as described previously [40].

2.7. Statistical analyses

The differences in community composition among all BDB batches were calculated using permutational multivariate analysis of variance (PERMANOVA) and visualized through non-metric multidimensional scaling (NMDS) ordination using metaMDS and as dendrograms using 'hclust' functions in the vegan package (v2.6.4) in R [41]. Differences in the bacterial alpha diversity and relative abundance of bacterial taxa among the 11 batches of BDB were compared using analysis of variance (ANOVA) on normal or log and square-root transformed data and a group-wise comparison was performed using Tukey's post hoc test. All p-values were adjusted using the Benjamini-Hochberg method. Adjusted p-values < 0.05 were considered statistically significant. To compare the body weight between mock-treated and BDB-treated mice over time, we employed a linear mixed-effects model (LMM) to account for the repeated measures nature of our data (body weight), as it considers both fixed effects (treatment group, time) and random effects (individual mice). The LMM was fitted using the lmer function from the lme4 package in R [42]. To assess the differences in the pulmonary health between mock and BDB-exposed mice, ordinal degree scores were compared using students *t*-test.

3. Results

3.1. Microbial composition of the BDB

The manufactured BDB batches were analysed for their microbial composition. DNA was extracted and the composition was analysed using 16S rRNA gene sequencing as described in the materials and methods (section 2.2). The microbial composition of all individual raw material components has been reported before [26] and the microbial composition of one of the lots from batch 10 was previously described in Nurminen et al. [29]. PERMANOVA revealed that the bacterial community composition was different among the 11 batches ($p = 0.001$). All batches had a high diversity as measured by Shannon's index, ranging from 5.4 to 6.6 (median) and observed ASVs were between 280 and 1200 (Fig. 1). Major differences in the diversity and richness were observed between the first two and the last batches compared to the others (Supplementary Table 2). Batch 8 had slightly higher intra-batch variation in Shannon's diversity index and richness (Fig. 1) but was within the overall variation among all BDB batches. Hierarchical clustering revealed that different lots of a single BDB batch clustered together with similar microbial composition for the first five batches compared to the remaining 6–11 batches (Fig. 2a). This was also observed in the NMDS, where the bacterial community compositions of the first five batches were different from the subsequent seven batches produced (Fig. 2b). Proteobacteria was the most abundant phylum in all BDB batches ranging from 31 % (batch 1) to 37.5 % (batch 11) followed by Bacteroidota (14–22 %) and Chloroflexi (8–14 %) (Fig. 3). A high variation in the relative abundance of bacterial phyla such as Proteobacteria, Bacteroidota and Acidobacteriota was observed among the BDB batches whereas less variation was observed for the relative abundance of Chloroflexi, Actinobacteriota and Verrucomicrobiota (Supplementary Table 2).

3.2. Inactivation of BDB

We next set out to neutralise any potential pathogens in the BDB to create a sterile product. To this end, we selected one BDB batch (batch 10) and autoclaved four individual samples from this batch at 121 °C or 134 °C, extracted DNA and RNA, and compared the presence of pathogens in autoclaved and untreated BDB.

We first analysed the presence of pathogenic human viruses and parasites commonly present in soil, namely adenovirus, enterovirus, rhinovirus, norovirus, sapovirus, parechovirus, influenza A virus, hepatitis A virus, Cryptosporidium and Giardia. We did not detect any of these pathogens even in the live BDB (Supplementary Table 3).

To enable the use of BDB in pre-clinical trials, the material was analysed according to the standardized FELASA specific pathogen list [37] (Table 2). We assessed the presence of rodent pathogens including bacteria, viruses, mites, and helminths through aerobic and anaerobic culture of autoclaved BDB as well as by PCR using DNA and RNA extracted from both live and autoclaved BDB (details in material and methods section 2.5). In BDB autoclaved at 121 °C, aerobic outgrowth of the environmental bacteria *Fictibacillus* spp., *Micrococcus luteus, Macrococcus brunensis, Neisseria subflava*, and two murine pathogens *Bacillus megaterium,* and *Staphylococcus* spp. was detected, while no bacterial outgrowth was detected in the anaerobic culture (Table 3). Likewise, Parvovirus DNA and pinworm (*Enterobius vermicularis*) DNA were detected through PCR analyses in both the live BDB and BDB autoclaved at 121 °C. We observed that after autoclaving at 134 °C, there was no outgrowth of any environmental or pathogenic bacterial species nor were bacterial, viral or parasitic nucleic acids detected (Table 3 and

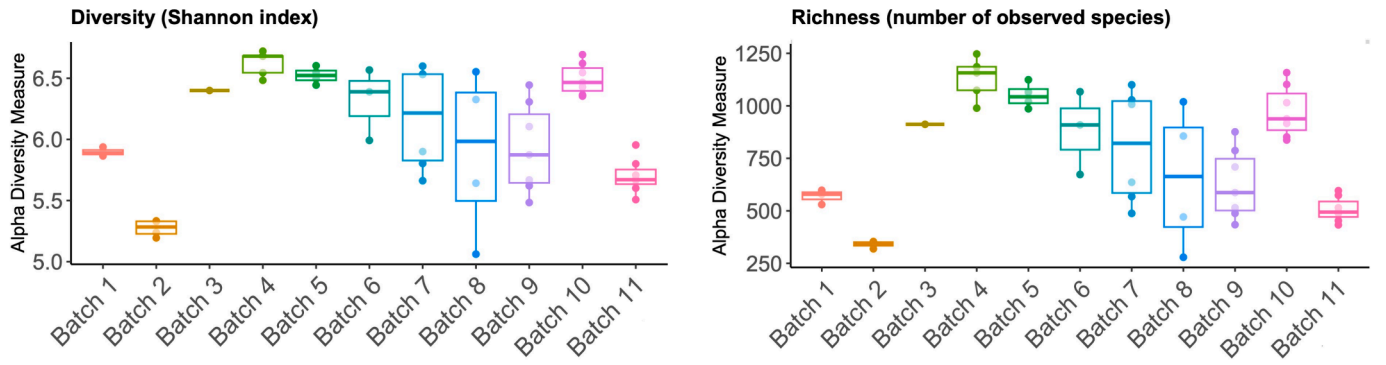

Fig. 1. BDB contains a high diversity and richness of bacteria. 16S rRNA sequencing was performed on DNA extracted from 11 batches of freshly produced BDB and alpha diversity of the identified bacteria was assessed using amplicon sequence variants (ASVs) from the sequence data. The diversity (Shannon index) and richness (number of bacterial species) were found to be consistently high in all 11 batches. The points in each box plot represent different lots of BDB sampled within the batch. Statistics are shown in Supplementary Table 2.

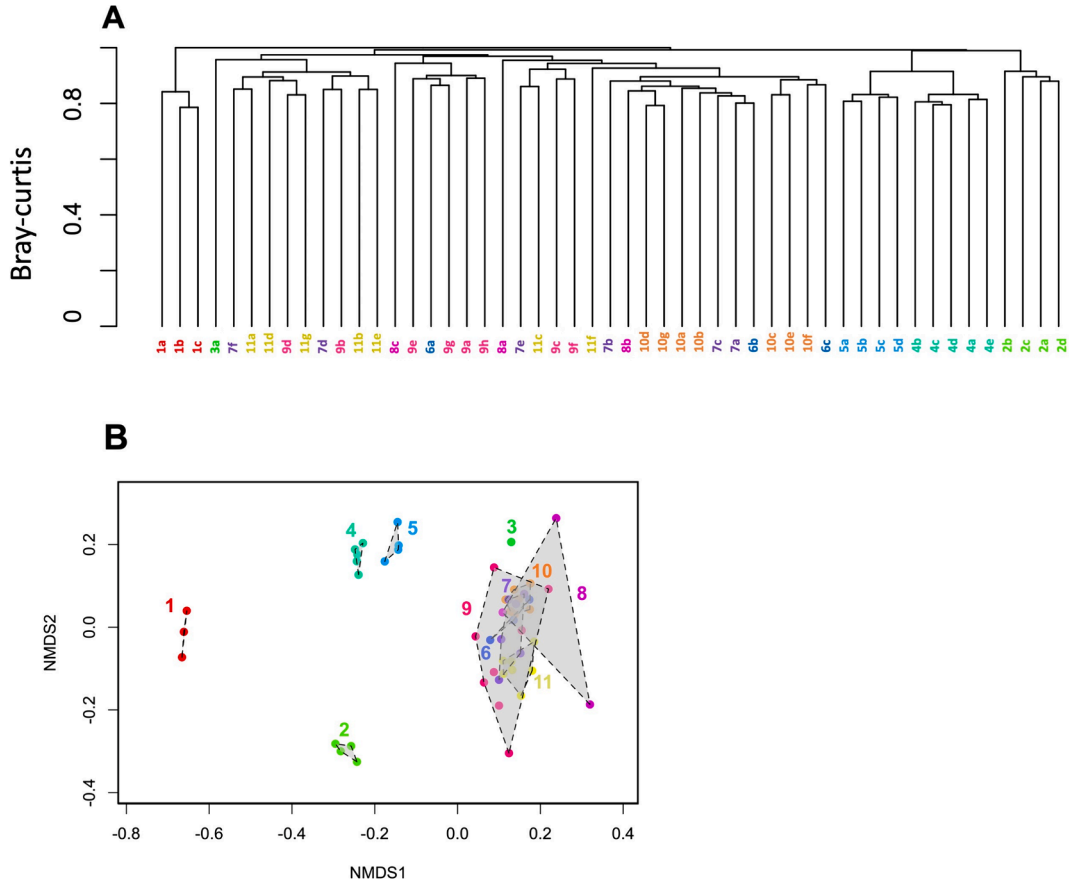

Fig. 2. The community composition of bacteria in the BDB batches. A. Hierarchical clustering based on group average linkage using bray-curtis dissimilarity metric was done for all samples analysed from 11 BDB batches using 'hclust' function in vegan package in R. The dendrogram is based on the relative abundance of ASVs and reveals the similarity or dissimilarity in ASV distribution across 11 batches of BDB. The alphabets represent the lots sampled within the batch. Batch one outgroups from the others, and a second bifurcation is seen between batch 2–5 and 6–11. B. Non-metric multi-dimensional scaling (NMDS) was done to visualize the community composition of bacteria within and between the batches based on relative abundance of ASVs using bray-curtis dissimilarity metric using metaMDS function in vegan package in R. The numbers represent the batches.

Supplementary Table 3). The reproducibility of the inactivation at 134 °C was confirmed by autoclaving additional BDB samples, which varying quantities of the BDB (data not shown).

3.3. Sterile BDB is safe to use in an experimental mouse model of type 1 diabetes

To assess whether exposure to inactivated BDB is safe in an experimental animal model prone to develop an immune mediated disorder, we exposed young (3 weeks of age) male and female NOD mice to BDB or mock exposed the animals (as described in section 2.6). We monitored

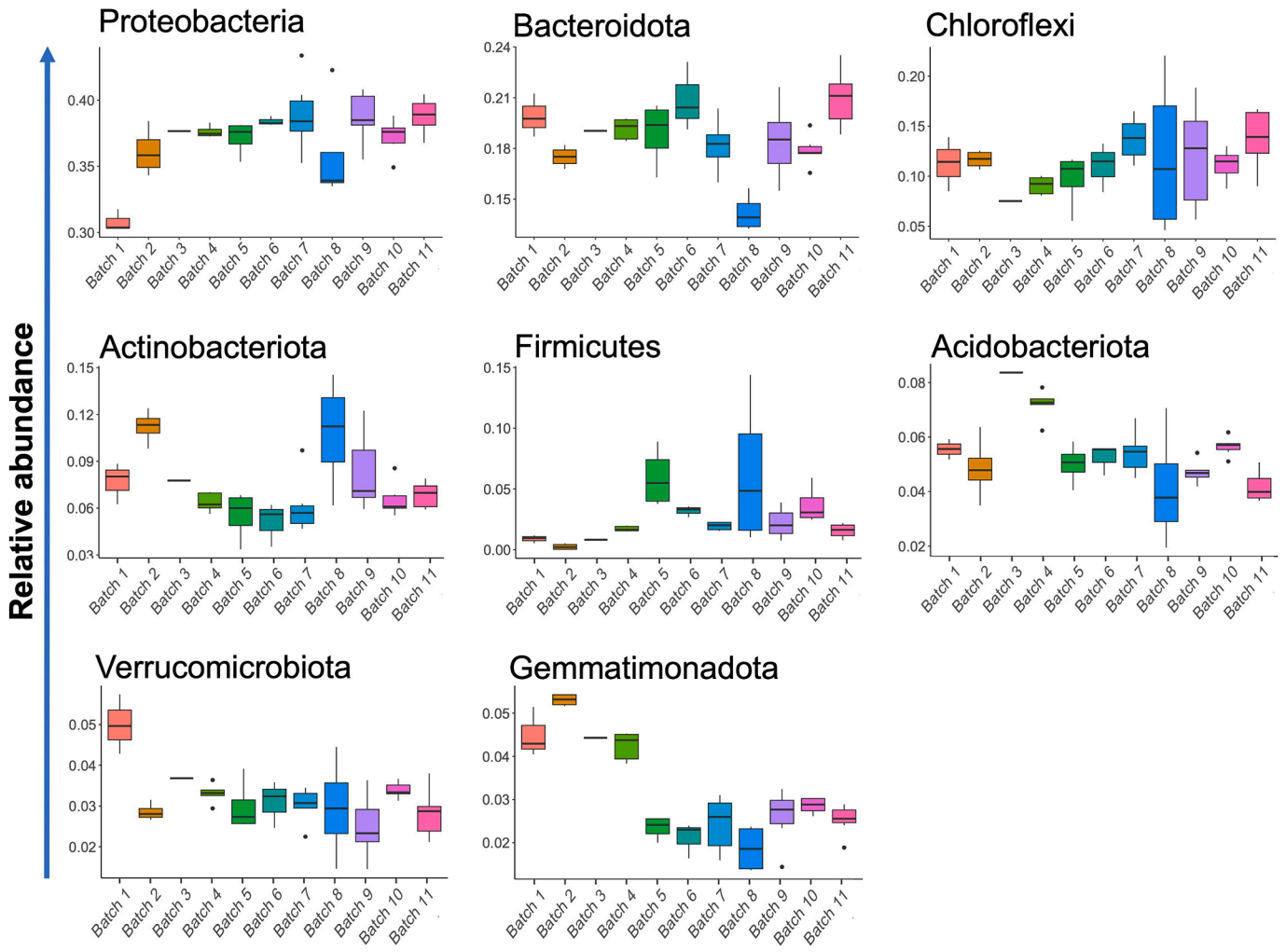

Fig. 3. The BDB comprises of a high abundance of bacterial phyla normally found in environmental samples such as soil and plants. The relative abundance of bacterial phyla from the 16S rRNA sequencing data was compared among the 11 batches of BDB. Phyla representing more than 1 % of the total bacteria are shown. The relative abundances of the most abundant bacteria were different whereas those of less bacteria were similar among the BDB batches (Statistics in Supplementary Table 2).

Table 3
Comparison of presence of murine pathogenic bacteria, viruses and parasites in live and BDB autoclaved at 121 °C or 134 °C.

	Bacteria			Virus	Parasites
Detection method	**Aerobic culture**	**Anaerobic culture**	**Nucleic acids**	**Nucleic acids**	**Nucleic acids**
Live BDB	Not tested	Not tested	None detected	Parvovirus DNA	*Enterobius vermicularis* DNA
BDB autoclaved at 121 °C	*Bacillus megaterium* and *Staphylococcus* spp.	None detected	None detected	Parvovirus DNA	*Enterobius vermicularis* DNA
BDB autoclaved at 134 °C	None detected	None detected	None detected	None detected	None detected

the effect of exposure to BDB on several health parameters including weight gain, animal appearance and behaviour. We observed that 30 min weekly (5 times per week) exposure to BDB was well-tolerated. Notably, the mice did not only move around in the BDB but also consumed it. We observed no adverse impact on weight gain during the exposure period or after the exposure was discontinued (Fig. 4). None of the animals developed diabetes (Supplementary Fig. 1). Pulmonary health of the animals was assessed at the endpoint and did not reveal any noticeable differences between exposed or mock exposed animals (Supplementary Fig. 2). We also analysed lung tissue for signs of inhaled particulate matter but none was detected.

4. Discussion

In this report, we describe the production of a blend with potential microbiome- and immune modulatory capacity. BDB was produced from commercially available soil and plant-based material made from easily accessible natural resources in Finland. The components have a well-characterized microbial composition [26] and were use in well-defined proportions. Soil is a large natural reservoir with high microbial diversity and the beneficial effects of microbiota on human health through direct and indirect means have been well documented [43,44,45]. Soils and plants from Northern Europe, particularly protected areas and other natural and seminatural ecosystems, are

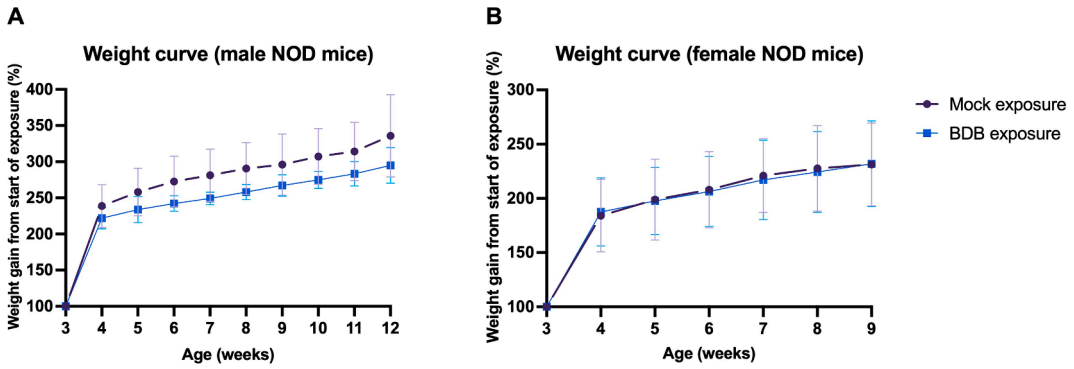

Fig. 4. BDB exposure had no adverse effect on weight gain in NOD mice. (A) Male NOD mice (age 3–4 weeks) were exposed to mock (n = 5) or BDB five times a week (n = 5)) followed by six weeks of observational period. (B) Female NOD mice (age 3–4 weeks) were exposed to mock (n = 8) or BDB five times a week (n = 9) for 6 weeks. Weight measurements were done weekly and shown relative to the weight at 3 weeks and compared between mock and BDB exposed mice using linear mixed-effects model (LMM). Data is presented as percentage weight gain, mean ± SEM.

relatively free from typical anthropogenic disturbances, such as pollution, irrigation, fertilization, invasive species, human trampling and droppings of domestic animals [46]. They are therefore close to being microbiologically and chemically pristine [47], which is why organic materials from those areas were chosen as the ingredients of the BDB. It is worth noting however, that natural phenomena such as seasonal variation, moisture, sunlight, soil pH, nutrient contents as well as anthropogenic disturbances such as deforestation, urbanization, land use change, agriculture etc. can alter the soil composition and microbiota considerably [25,43,48]. As such, we acknowledge that the components of BDB that are produced in places that differ in terms of climate and land use may not be microbiologically identical, despite our established protocol.

We undertook a series of steps to produce a powder-like consistency that allows us to incorporate the BDB into a broad range of formulations intended for human clinical or animal (pre-clinical) trials. We have previously evaluated the efficacy of the BDB in modulating the composition of commensal microbiota and immune functions via topical application through hand rubbing (mainly suitable for adults [29,30]) or by mixing in sandboxes (targeting children in daycare [28]). We observed that topical exposure was safe and could modify the skin microbiota and potentially the gut microbiota which probably occurred through (accidental) ingestion as well as inhalation, the latter being more probable due to the powder like consistency of BDB.

We observed that the BDB had a very high number of bacterial species (richness) and a high diversity. However, there was some inter-batch variations in the alpha diversity as well as the relative abundance of bacterial phyla. This could be due to insufficient blending or the length of time the ingredients were stored for since the production technique and storage conditions remained unaltered. We have previously shown that long term storage of the BDB components alters the relative abundance of several bacterial taxa [26]. Indeed, we observed that there was a high variation in the relative abundance of major bacterial phyla (Fig. 3) among the BDB batches although the diversity and richness were not largely compromised. This difference in the relative abundance of bacterial taxa could perhaps raise concerns about the ability of different batches of BDB to reproducibly modulate the microbiome or immune function. Emerging evidence suggests that exposure to diverse microbial communities and their associated antigens from the environment fosters immunoregulation [12,13,14]. Thus, it is plausible to hypothesize that minor fluctuations in the relative abundance of bacterial taxa in the BDB may not significantly impact its effectiveness, provided that microbial diversity and richness are maintained at high levels. A high microbial diversity and richness may offer a simple yet potent strategy to enhance microbial exposure, particularly for urban populations who are often deprived of sufficient microbial interactions due to urbanization and limited exposure to natural biodiversity [21,48,49]. Nevertheless, certain bacterial taxa or groups have documented associations with immune function and overall health [50,51], warranting further investigation in future studies to fully elucidate these relationships.

Live BDB may contain potential pathogens. This is not surprising since soil in general harbors a vast variety of microbes, many of which may be potentially pathogenic including *Bacillus anthracis*, *Clostridium tetani*, and *Clostridium botulinum*, as well as antibiotic-resistant Staphylococci. However, in our previous studies, exposure to BDB did not induce adverse health effects in healthy individuals [28,29,30,31,52]. In fact, children in daycare centers are in daily contact with many sources of potential pathogens, including those carried by other children and others present in daycare yards. The added exposure to BDB in, for example, sandboxes was not associated with infections or other side effects [20,22,28,53]. This is in line with reports suggesting that exposure to soil pathogens can in fact promote immune tolerance and a better functioning immune system [44,45,54]. In this context, is may be of relevance that the manufacturing process of BDB involved a careful mixing that effectively homogenized the blend thus breaking clusters of conspecific bacteria, including potential pathogens. Furthermore, we always used the BDB in freeze-dried form that prevents the outgrowth of any potential pathogens that may be present in low relative abundance. Future exposure studies, particularly those involving human subjects, should include thorough pathogen screening and, when utilizing inactivated BDB, careful validation of sterility to ensure safety.

The use of live BDB could be a problem if it is intended for use in immunocompromised and/or chronically ill populations. Exposure to less virulent pathogens may cause opportunistic infections in such individuals [55,56]. This also applies to animals, particularly disease models of immune-mediated disorders. In addition, the topical application of live BDB may pose a problem in subjects with a broken skin barrier due to cuts and wounds for instance. A potential solution to this is to use sterile BDB. We therefore inactivated the freeze-dried BDB and observed that autoclaving at 134 °C was effective in removing known human and murine pathogens. In an initial safety assessment, we used experimental mice to evaluate the effects of inactivated BDB. Notably, we observed no adverse health effects, including weight loss, in the mice exposed to BDB, even after consumption. These findings suggest that exposure to inactivated BDB is not associated with negative side effects.

The dose (50 ml) of inactivated BDB, frequency (5 times a week) and duration (30 min per exposure for six weeks) of exposure represent an arbitrary exposure regimen and it remains to be seen whether autoclaving followed by such treatment change the potential of BDB to alter skin and gut microbiota, stimulate PRRs and promote the production of metabolites that can modulate immune function. A chemical analysis of sterile BDB could provide information on its content of immunoregulatory components. Notably, bacterial endotoxins, including

lipopolysaccharides (LPS), that are major antigenic determinants of gram-negative bacteria are resistant to autoclaving and heat treatments [57]. In addition, our previous study revealed that BDB autoclaved at 121 °C exhibited immunoregulatory potential in C57BL/6 mice and stabilized the microbiome, which was characterized by less dramatic changes in the gut microbiome of BDB exposed mice compared to mock-exposed mice over a period of three weeks [39]. However, here we observed that autoclaving at 121 °C does not effectively remove all murine pathogens suggesting that autoclaving at 134 °C is a better way to sterilize the product. Collectively, our studies demonstrated that exposure to BDB was safe in mice. Building on this evidence, we suggest that the safety of the topical administration of inactivated BDB can also be evaluated in immunocompromised humans.

We observed that proteobacteria was the major bacterial phyla in all of the BDB batches we have produced so far. Taxa belonging to Proteobacteria are known producers of endotoxins, which could potentially raise concerns about the potential pathogenicity associated with the use of BDB. However, Protoebacteria is also a major component of skin microbiota and is associated with innate and adaptive immune responses [13,20,58,59]. Importantly, we have observed in several of our studies that exposure to non-inactivated (live) BDB is safe in human subjects and animal models [20,29,39,60]. Moreover, lipopolysaccharides (LPS), which are the endotoxins that are produced by Proteobacteria are known immunomodulatory molecules [61,62,63]. As such, there is ample evidence to suggest that the higher abundance of Proteobacteria in the BDB is not necessarily bad and may actually be beneficial.

A limitation of our study is that we analysed the bacterial composition of the BDB in detail using amplicon sequencing and but did not assess additional microbes. Fungi, viruses and other microbes could also play a role in modulating human commensal microbiota composition and induce immune regulation. Bacteria are however the most well-studied microbes in terms of immunomodulation and therefore we focused on bacteria in our current study. More detailed analyses of microbial composition, including species-level data specially on potentially pathogenic species through metagenomic sequencing, could offer enhanced understanding of interactions between the immune response and microbiome and as such should be explored in future studies.

5. Conclusions

In this report, we describe the production, microbial analyses and safety aspects of a soil and plant derived natural product, which has been shown to have microbiome and immune modulatory capacity in previous studies. We show that the alpha diversity of environmental bacteria remained consistently high among the BDB batches produced however with some considerable alterations in the relative abundance of some bacterial taxa. We also show that the BDB is free of specific human pathogens and autoclaving at 134 °C inactivated potential human as well as murine pathogens. We found that BDB can be easily sprinkled over cage bedding material and topical exposure through the cage bedding was well-tolerated by immune-mediated disease-prone mice. Remarkably, these mice even consumed the BDB without experiencing any side-effects.

The use of sustainable and safe natural ingredients for the prevention or treating immune-mediated health disorders presents an attractive proposition. However, whether exposure to live or inactivated BDB has health benefits, with or without altering gut microbiota composition, remains to be determined. Future studies are needed to establish the safety and potential health benefits associated with BDB exposure, as well as its impact on disease risk. The powder-like form of the BDB allows flexibility for its incorporation into various formulations, suitable for different routes of administration for potential preclinical and clinical trials. These formulations may include topical applications and combination into lotions intended for topical use in infants (Clinical trial: NCT03872219).

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: NN, SO, OHL, HH and AS were named as inventors and AS has received royalties from a patent EP3551196 A1 'immunomodulatory compositions', originally submitted by University of Helsinki. OHL, HH and AS belong to the founders and members of the board of Uute scientific Ltd., a start-up launched by University of Helsinki, which develops solutions for cosmetic, textile, and plasticine industry. MIR. and AS have been named as inventors in a patent application 'Immunomodulatory gardening and landscaping material' submitted by University of Helsinki (Patent application number 20175196 at Finnish Patent and Registration Office)..

Data availability

Data availability is included in the manuscript file

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2024.09.011.

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