

Physiological Responses of Adults during Soil-mixing Activities Based on the Presence of Soil Microorganisms: A Metabolomics Approach

Seon-Ok Kim

Department of Bio and Healing Convergence, Graduate School, Konkuk University, Seoul 05029, Republic of Korea

Su Young Son and Min Ji Kim

Department of Bioscience and Biotechnology, Konkuk University, Seoul 05029, Republic of Korea

Choong Hwan Lee

Department of Bioscience and Biotechnology, and Research Institute for Bioactive-Metabolome Network, Konkuk University, Seoul 05029, Republic of Korea

Sin-Ae Park

Department of Bio and Healing Convergence, Graduate School, and Department of Systems Biotechnology, Konkuk University, Seoul 05029, Republic of Korea

ADDITIONAL INDEX WORDS. electroencephalogram, heart rate, horticultural activity, horticultural therapy, metabolite profiling, *Mycobacterium vaccae*

ABSTRACT. *Mycobacterium vaccae* is a species of nonpathogenic bacterium that lives naturally in soil. This study compared the physiological effects at a metabolomic level with autonomic nervous system responses in adults during soil-mixing activities, based on the presence or absence of *M. vaccae* in the soil. Twenty-nine adult participants performed soil-mixing activities for 5 minutes using sterilized soil with culture media and *M. vaccae*, respectively. Blood samples were drawn twice from each participant after each activity. Electroencephalograms and electrocardiograms were measured during the activity. Serum metabolites underwent metabolite profiling by gas chromatography, followed by multivariate analyses. Soil-emitted volatile organic compounds were identified using the solid-phase microextraction and gas chromatography-mass spectroscopy, followed by multivariate analyses. The volatile compound analysis revealed that the metabolites related to esters and sulfur-containing compounds are greater in soil with *M. vaccae*. Serum metabolomics revealed that the treatment group (soil inoculated by *M. vaccae*) possesses relatively higher levels of inter-alia organic and amino acids compared with the control group (soil mixed with culture media). In the treatment group, the electroencephalogram and electrocardiogram revealed that alpha band activity of the occipital lobe increases, while heart rate decreases. This study concludes that *M. vaccae* soil contact can affect human metabolic and autonomic reactions.

By 2050, ≈70% of humans are expected to live in urban areas (Dye, 2008). Urbanization has exposed humans to more artificial elements. Continuous exposure to these environments exacerbates human stress levels. Increases in stress levels caused by urban life could lead to mental health problems, such as mood and anxiety disorders (Peen et al., 2010) and schizophrenia (Krabbendam and van Os, 2005; Pedersen and Mortensen, 2001). Owing to the negative effects of urban life, people's interests in human health, well-being, and nature have recently increased.

Humans feel an instinctive preference for nature, and Orians' Savannah theory and Wilson's biophilia hypothesis support this (Orians, 1986; Wilson, 1984). Ulrich (1983) reported that stress from the external environment can be reduced through exposure to nature.

Previous studies have shown that staring at green plants stabilizes the human autonomic nervous system and activates the alpha wave frequency of brain waves, leading to physiological and psychological relaxation (Ikei et al., 2014; Park et al., 2016). In addition, when performing work activities in an environment with plants, the sympathetic nerve activity and oxyhemoglobin of the worker's left frontal cortex is reduced, resulting in physiological relaxation (Park et al., 2017). Park et al. (2020) performed metabolite profiling to investigate the effects of horticultural interventions on cognitive improvements in older adults; they reported that horticultural activities have psychological and cognitive health effects by enhancing tryptophan and serotonin in the serum of elderly individuals.

As such, various effects in physiological and psychological aspects that are obtained from interactions with nature have been reported, but prior studies have focused on green plants, predominantly in the form of holistic and synergistic approaches that combine plants and soil. To fully understand the healing mechanism of horticultural activities, we must now study not only the effectiveness

Received for publication 20 Oct. 2021. Accepted for publication 23 Feb. 2022. Published online 26 April 2022.

This work was performed with the support of the National Research Foundation of Korea grant funded by the Korean government (Project title: Psychological and emotional control effects and mechanisms of plant and soil-derived metabolites in horticultural activity; Project No. 2019R1F1A105441113). This paper was supported by the KU Research Professor Program of Konkuk University.

S.-O.K. and S.Y.S. contributed equally to this work.

S.-A.P. is the corresponding author. E-mail: sapark42@konkuk.ac.kr.

This is an open access article distributed under the CC BY-NC-ND license (<https://creativecommons.org/licenses/by-nc-nd/4.0/>).

Table 1. Inclusion and exclusion criteria for the experimental participants and precautions before participating in the experiment to investigate the effects of the *Mycobacterium vaccae* soil on the physiological responses of humans during the soil-mixing activity.

Inclusion criteria	<ul style="list-style-type: none"> ● No previous psychopathological diseases. ● Not left-handed. ● No previous cardiovascular diseases. ● No previous olfactory function-related disorders. ● Not pregnant, not lactating, and not menstruating women.
Precautions before participating in the study	<ul style="list-style-type: none"> ● No alcohol consumption the day before the experiment. ● No physical activity (e.g., breathless high-intensity physical activity for more than 60 min) on the day before the experiment. ● No smoking within 3 h before the experiment. ● No caffeine consumption within 3 h before the experiment. ● No application of cosmetics with strong scents, such as perfumes and sprays, on the day of the experiment.

through a complex approach but also the role of each plant and soil. There are currently few studies related to the mechanism of healing on the human body through soil contact.

Examining the effects of urban environmental biodiversity on commensal microbiome and immunoregulation in children, biodiversity interventions such as contact with plants and soil enhanced immunoregulatory pathways, as were associated with increased plasma transforming growth factor beta 1 levels and proportion of regulatory T cells (Roslund et al., 2020). In addition, with long-term follow-up of more than 1 year, biodiversity interventions enriched the commensal microbiome and suppressed the potentially pathogenic bacteria on the skin, including taxa related to immune regulation (Roslund et al., 2021).

Mycobacterium vaccae belongs to the Actinomycetales and are a nonpathogenic bacterial species that live naturally in the soil. They are predominantly found in soil, water, and mud (Hoisington et al., 2015), and are reported to have a strong immunomodulatory effect, according to the hygiene hypothesis in which the immune system was developed by exposure to various microorganisms (Rook et al., 2004). In rodents (Rodentia), exposure to *M. vaccae* has been reported to activate their immune response, thereby reducing inflammation and stress-induced behavioral disorders and improving learning abilities (Fonken et al., 2018; Frank et al., 2018; Reber et al., 2016; Smith et al., 2019). Furthermore, this immune activation promoted the activation of serotonin neurons, thereby increasing the level of serotonin and reducing anxiety in rodents (Lowry et al., 2007). With similar results, O'Brien et al. (2004) reported that when *M. vaccae* is injected into patients with lung cancer, the patients' sense of happiness and vitality increases. However, there are few studies examining the effect of *M. vaccae* in clinical trials other than intracorporeal injections or oral administration on humans.

To understand the therapeutic mechanism of horticultural activities and nature-based therapy clearly, studies on the effects of interactions with soil, soil microorganisms, and plants are necessary. Therefore, this study measures the effect of a soil-mixing activity on the psychological and physiological responses of humans, according to the presence or absence of *M. vaccae* microorganisms in the soil. Depending on the presence or absence of *M. vaccae* microorganisms in the soil, the effects of a soil-mixing activity on human metabolic and autonomic reactions will be different.

Materials and Methods

PARTICIPANTS. Twenty-nine adults from 20 to 59 years old (8 men, 21 women; average age 28.6 ± 9.8 years) participated in this

study. Participants were recruited using a convenience sampling method. A flyer that included study information was distributed to apartments and churches in Gwangjin-gu, Seoul, Republic of Korea. Participants were recruited according to the inclusion and exclusion criteria shown in Table 1, so as not to influence other physiological data. Before conducting the experiment, participants were informed of the research contents and precautions, and written consents were obtained before participation in the research. To collect participants' demographic information, age, sex, height, weight, and body mass index using a body composition analyzer (ioi 353; Jawon Medical, Gyeongsan, Republic of Korea) were recorded. Participants received the equivalent of \$10 as an incentive to complete the experiment. This study was approved by the Bioethics Committee of Konkuk University, Seoul, Republic of Korea (7001355-201911-HR-345).

PREPARATION OF THE SOIL SAMPLE. *M. vaccae* KCTC 19087 was obtained from the Korean Collection for Type Cultures (KCTC, Jeongseup, Republic of Korea). *M. vaccae* was cultivated on tryptic soy broth for 4 d at 37 °C in the dark and by shaking (200 rpm). For soil sample preparation, soil samples were autoclaved at 121 °C for 15 min. The sterile soil (1.5 g) was mixed with 2.5 mL of sterile water, a tryptic soy broth, and a 4-d cultured *M. vaccae* strain [1.35×10^9 colony-forming units (cfu)/mL], for 2 d at 37 °C to obtain various types of volatile organic compounds (VOCs). After incubation, the samples were transferred to a gas chromatography–time-of-flight–mass spectrometry (GC-TOF-MS) instrument to analyze VOCs.

Microbial-treated soil was mixed with sterilized peatmoss (2000 mL), perlite (800 mL), and water (200 mL), with a 50 mL *M. vaccae* medium that was cultured for 4 d (1.35×10^9 cfu/mL). The control soil was mixed with 50 mL of cultured medium that did not contain microorganisms.

EXPERIMENTAL CONDITIONS. This study was conducted in an experimental space (180 cm × 200 cm) in Konkuk University. In the experimental space, there was a desk (180 cm × 90 cm) on which a basin of soil could be placed, and the distance between the basin and the participants was set at 50 cm. To minimize external visual stimulation, white hardboard paper was placed before the desk, and ivory-colored curtains were installed on either side of it. The environmental conditions measured by thermo-hygrometer (O-257; DRETEC Co., Kawaguchi, Japan) of the space during the experiment were as follows: temperature 26.5 ± 1.8 °C, relative humidity $43.3\% \pm 15.8\%$.

EXPERIMENTAL PROCEDURE. This study was conducted through a single-blinded experiment and randomized crossover study

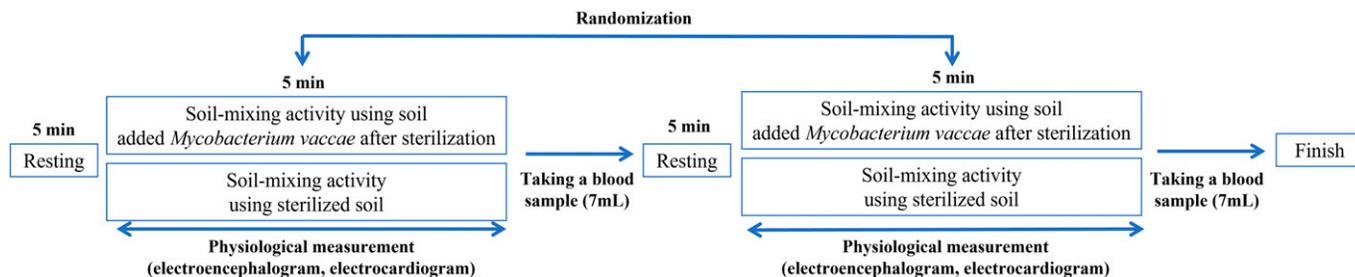


Fig. 1. Experiment protocol used in this experiment to investigate the effects of the *Mycobacterium vaccae* soil on the physiological responses of humans during the soil-mixing activity.

method. To investigate the effect of the *M. vaccae* soil on the physiological responses of humans during the soil-mixing activity, an experiment was performed according to the procedure shown in Fig. 1. Before performing the soil-mixing activity, participants were asked to look at the white wall in front of them for 5 min to encourage relaxation. Thereafter, they mixed the soil in the basin with both hands for 5 min. After the soil-mixing activity, 7 mL of blood was collected for the metabolome analysis. After the first trial, participants performed the other activity with the procedure mentioned previously following a 5-min break, and the trial order was randomly assigned. The duration of the entire experiment was ≈ 40 min per participant.

PSYCHO-PHYSIOLOGICAL MEASUREMENT. To compare the physiological responses of participants when performing soil-mixing activities according to the presence or absence of *M. vaccae*, electroencephalography (EEG) and electrocardiography (ECG) were measured using a wireless dry EEG device (Quick-20; Cognionics, San Diego, CA) and a medical electrode (HP-OP42; Hurev, Wonju, Republic of Korea), respectively. The dry wireless EEG device used in this study minimizes the risk of electrical stimulation by using a dry electrode. It also can be detached quickly if the participant feels uncomfortable.

Data were collected by amplifying and processing electrical signals measured through dry electrodes applied to the scalp. The device is safety certified by the European Commission and Federal Communications Commission (Kim et al., 2020). The electrode application complied with the international 10- to 20-electrode arrangement system (Jasper, 1958). The reference electrode was attached to the left earlobe (A1). According to the international electrode method, this study performed EEG monitoring at the left occipital cortex (O1) and right occipital cortex (O2). Previous studies have reported that EEG could improve our understanding of brain activity and human central nervous system activity through olfactory stimulation (Lorig, 1989). Furthermore, it has been reported that cortical activity can be enhanced by a significant increase in fast alpha activity in bilateral posterior regions of the brain through olfactory stimulation (Iijima et al., 2009). Masago et al. (2000) reported that the occipital and temporal lobes are involved in integrated sensory information processing, including the sense of smell, and are associated with complex and integrated neural activity related to odors thought to occur in these areas. In addition, as serotonin is a major neurotransmitter involved in the occipital lobe (UKEsays, 2020), which also regulates human emotions and mood, the occipital lobe was selected as the measurement cortex to investigate the effect of the soil-mixing activity on human mood, based on the presence or absence of *M. vaccae*. The ECG

electrodes were placed at the end of the right collarbones and left rib bones of participants.

MEASUREMENT OF BRAIN-DERIVED NEUROTROPHIC FACTOR. Blood samples were collected to measure variations in the brain-derived neurotrophic factor. The participant's specimens were collected by a skilled sampler, each of whom was a professional nurse. The blood samples were collected in a clot activator tube (Vacutainer 367896; BD Diagnostics, Franklin Lakes, NJ). The collected blood was kept at room temperature for 20 min and centrifuged for 10 min at $1000 g_n$ to separate the serum samples. Thereafter, the aliquot was stored at -70°C in a deep freezer. The enzyme-linked immunosorbent assay kits were used to measure brain-derived neurotrophic factor (BDNF; AbCAM, Cambridge, UK) according to the manufacturer's instructions.

EXTRACTION FOR VOCs USING HEADSPACE SOLID-PHASE MICROEXTRACTION. The headspace solid-phase microextraction (HS-SPME) was performed for three biological replicates of soil-treated distilled water (S), culture media (SM), and soil inoculated with *M. vaccae* strain (SMV) samples to obtain VOCs. Each soil sample (1.5 g) was mixed with 2.5 mL of distilled water, culture media, and the *M. vaccae* strain. The sample mixtures were transferred into a 20-mL SPME vial and pre-incubated for 2 h. The HS-SPME of VOCs from soil samples were immediately performed using carboxen/polydimethylsiloxane/divinylbenzene (CAR/PDMS/DVB)-coated SPME fibers [75 μm (Supelco; Sigma-Aldrich, St. Louis, MO)] and collected by exposing the fiber to soil samples for 50 min at 37°C with 250 rpm. Following the extraction, the fiber was inserted into the GC injector (230°C) for the desorption procedure for 1 min. We followed the method of Singh and Lee (2018) for the extraction for VOCs with few modifications.

EXTRACTION FOR SERUM METABOLITE. Each human serum (200 μL) was extracted with cold methanol (1 mL) and 10 μL of an internal standard (1 $\text{mg}\cdot\text{mL}^{-1}$ 2-chlorophenylalanine) using a mixer mill (MM400; Retsch, Haan, Germany) at a frequency of 30 Hz for 10 min, with sonication for another 10 min. After homogenization, the suspension was stored at 20°C for 60 min. It was then centrifuged at $13,250 g_n$ for 10 min at 4°C (Centrifuge Universal 320; Hettich, Tuttingen, Germany). The supernatant was filtered through a 0.2- μm polytetrafluoroethylene filter (Chromdisc, Daegu, Republic of Korea). The filtered samples were dried completely using a speed vacuum concentrator (Biotron, Seoul, Republic of Korea). The final concentration of each sample was adjusted to $10 \text{ mg}\cdot\text{mL}^{-1}$ for the MS analysis. The serum extraction procedure was based on our previous research (Park et al., 2020).

Table 2. Descriptive information of participants who participated in the experiment to investigate the effects of the *Mycobacterium vaccae* soil on the physiological responses of humans during the soil-mixing activity (N = 29).

Gender	% (N)
Male	27.6 (8)
Female	72.4 (21)
Variable	mean ± SD
Age (years)	28.6 ± 9.8
Height (cm)	165.2 ± 6.9
Body weight (kg)	59.6 ± 11.4
Body mass index (kg·m ⁻²) ^z	21.8 ± 3.5

^zBody mass index = weight (in kilograms)/height² (in square meters).

ANALYSIS OF VOCs. The GC-TOF-MS instrument involved in the analysis of VOCs was identical to our previous study (Park et al., 2020). The GC analytical program for VOCs was programmed as follows: initially maintained at 33 °C for 3 min and elevated to 180 °C at a rate of 10 °C·min⁻¹. Finally, the temperature was raised to 240 °C at a rate of 40 °C·min⁻¹, for a duration of 4 min. The flow rate of helium was 1.5 mL·min⁻¹. The mass spectrum was collected with the mass range of 50–500 *m/z* at a rate of 10 scans per second.

ANALYSIS OF SERUM METABOLITES. The GC-TOF-MS analysis was performed as described previously by Park et al. (2020). The derivatized samples (1 µL) were injected into the GC-TOF-MS instrument in the splitless mode. The analytical program and parameter setting for analysis were adapted from our previous study (Park et al., 2020). The analytical samples were randomized in each block to reduce the effects of systematic errors.

DATA PROCESSING AND ANALYSIS. The measured EEG and ECG data were analyzed using the Bio-scan analysis program (Bio-Tech, Daejeon, Republic of Korea). The collected EEG raw data were analyzed using power spectrum analysis to identify the relative fast alpha (RFA) power spectrum and spectral edge frequency as 50% of alpha (ASEF50) (Sowndhararajan et al., 2015). ECG data were converted to heart rate variability (HRV) after high-pass filter preprocessing to obtain the average heart rate, low frequency band (LF), high frequency band (HF), and standard deviation of NN interval (SDNN).

The MS data processing and multivariate statistical analysis were performed as previously described (Park et al., 2020). For MS data processing, raw data derived from GC-TOF-MS were converted into a netCDF (*.cdf) format using an MS data system (ChromaTOF ver. 4.44; LECO Corp., St. Joseph, MI). Subsequently, the peak alignment, peak detection, peak normalization, and retention time were determined using MetAlign software

(RIKILT-Institute of Food Safety, Wageningen, The Netherlands). The results of alignment data were exported to spreadsheet files (Microsoft Excel, Office 2007; Microsoft, Redmond, WA). Multivariate statistical analysis was performed using software (SIMCA-P+ version 12.0; Umetrics, Umeå, Sweden). Principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed to compare different VOCs and serum metabolites. The significance of the PLS-DA and OPLS-DA models were defined by an analysis of variance testing of cross-validated predictive residuals (CV-ANOVA) using the SIMCA-P+ program. Different metabolites were selected by variable importance in the projection (VIP) value of the PLS-DA and OPLS-DA models. The discriminated metabolites were identified through comparing their retention time and mass fragment data (through MS) to the available databases, such as the Human Metabolome Database (2021), the National Institute of Standards and Technology database (version 2.0, 2011; FairCom, Gaithersburg, MD), Wiley 9 database (Wiley-VCH, Weinheim, Germany), and our in-house library of standard compounds.

Processed EEG and ECG data were analyzed by the Wilcoxon signed-ranks test, which was performed using statistical analysis software (IBM SPSS Statistics version 25 for Windows; IBM Corp., Armonk, NY). The significance level was set at *P* < 0.05. To analyze demographic information, descriptive statistics were performed on the mean, standard deviation, and percentage of each collection item using spreadsheet software (Microsoft Excel, Office 2007). Furthermore, the significantly different metabolites from the experimental groups were evaluated through Student's *t* test and one-way ANOVA, coupled with the Pearson's correlation coefficient between serum metabolites and the phenotypes, using statistical analysis software (IBM SPSS Statistics version 18 for Windows).

Results

DEMOGRAPHIC CHARACTERISTICS. The characteristics of participants of this study are summarized in Table 2. Twenty-nine adults from 20 to 59 years old (8 men and 21 women; mean age 28.6 ± 9.8 years) participated.

PSYCHO-PHYSIOLOGICAL RESPONSES. From the comparison of the EEG during the soil-mixing activity (based on the presence or absence of *M. vaccae* in the soil), the RFA and ASEF50 of the right occipital lobe were significantly higher during the soil-mixing activity that included *M. vaccae* (*P* < 0.05) (Table 3). As a result of comparing the HRV of participants during the soil-mixing activity (according to the presence or absence of

Table 3. Results of relative fast alpha power spectrum (RFA), spectral edge frequency 50% of alpha (ASEF50) by electroencephalography, according to the presence and absence of *Mycobacterium vaccae* in the soil during the soil-mixing activity.

	RFA ^z		ASEF50 ^y	
	O1	O2	O1	O2
Soil-mixing activity	Mean ± SD			
Using soil added <i>M. vaccae</i> after sterilization	0.055 ± 0.010	0.056 ± 0.009	10.398 ± 0.195	10.379 ± 0.221
Using sterilized soil	0.053 ± 0.009	0.053 ± 0.008	10.331 ± 0.169	10.302 ± 0.223
<i>P</i> value	0.062	0.024*	0.070	0.021*

^zRFA was calculated by [fast alpha (11–13 Hz power)]/[total frequency (4–50 Hz power)]; O1 = left occipital lobe; O2 = right occipital lobe.

^yASEF50 is the area from 8 to 13 Hz, which occupies 50% of the area in the entire frequency range.

**P* < 0.05 by the Wilcoxon signed-ranks test.

Table 4. Results of heart rate variability by electrocardiogram, according to the presence and absence of *Mycobacterium vaccae* in the soil during the soil-mixing activity.

Soil-mixing activity	Heart rate	LF ^z	HF ^z	SDNN ^z
	Mean ± sd			
Using soil added <i>M. vaccae</i> after sterilization	79.11 ± 22.52	0.48 ± 0.12	0.52 ± 0.12	71.78 ± 46.35
Using sterilized soil	87.00 ± 21.28	0.44 ± 0.11	0.56 ± 0.11	68.55 ± 52.24
<i>P</i> value	0.031*	0.092	0.092	0.362

^zLF (low frequency) = [low frequency band (0.04–0.15 Hz)]/[total frequency band (0.04–0.4 Hz)]. HF (high frequency) = [high frequency band (0.15–0.4 Hz)]/[total frequency band (0.04–0.4 Hz)]. SDNN = standard deviation of RR intervals (where R is a point corresponding to the peak of the QRS complex of the electrocardiography wave).

**P* < 0.05 by the Wilcoxon signed-ranks test.

M. vaccae in the soil), heart rate was significantly lower during the soil-mixing activity that included *M. vaccae* (*P* < 0.05) (Table 4). There were no significant differences in LF, HF, and SDNN between the two conditions (*P* > 0.05).

NONTARGETED VOLATOLOME PROFILING OF SOIL SAMPLES. The disparities of VOCs in various soil samples were identified, including the soil of S, SM, and SMV. These were evaluated

using multivariate analysis of SPME-GC-TOF-MS datasets. As shown in Fig. 2, the PCA score plot based on SPME-GC-TOF-MS data showed a marked distinguishment with different soil samples by PC1 (45.48%) and PC2 (24.31%) (Fig. 2A). Moreover, the PLS-DA showed a similar pattern to the PCA score plot (Fig. 2B). The statistical parameters of PLS-DA models were evaluated by R²X (0.697), R²Y (0.987), Q² (0.959),

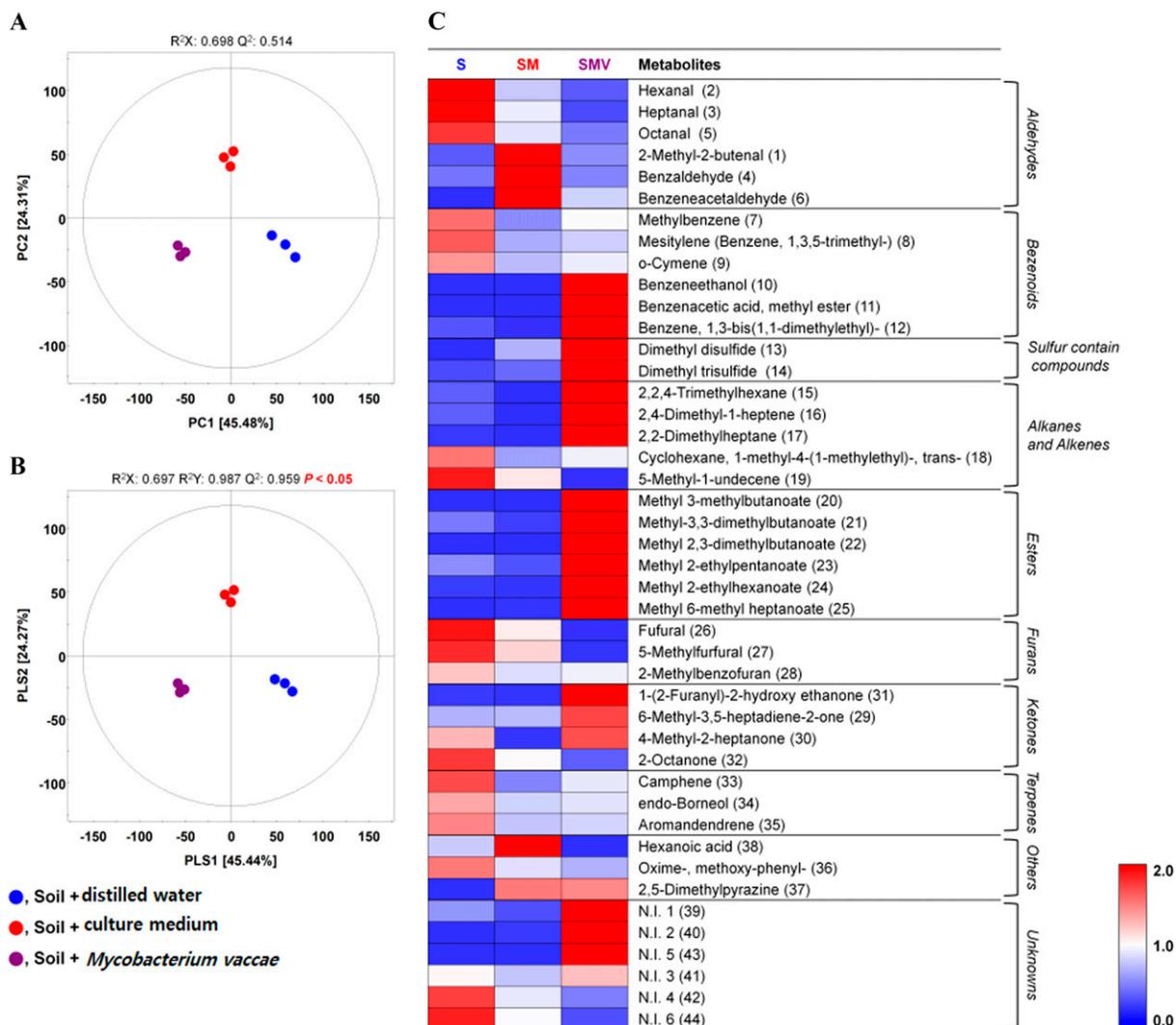


Fig. 2. (A) Principal component analysis and (B) partial least-square discriminant analysis score plot derived from solid-phase microextraction gas chromatography–time-of-flight–mass spectrometry datasets for various soil samples; soil treated with distilled water [S (λ)]; soil treated with culture media [SM (λ)]; soil inoculated with *Mycobacterium vaccae* [SMV (λ)]. (C) Heat map analysis for the relative abundance of different volatile organic compounds (variable importance in the projection > 1.0, *P* < 0.05) derived from the gas chromatography–time-of-flight–mass spectrometry analysis. The colored squares (blue to red) indicate fold changes that are normalized by the average of each metabolite; N.I. = nonidentified.

and CV-ANOVA P value ($P < 0.05$). This indicated the model validation, fitness, and prediction accuracy, as shown in the Fig. 2. Based on the PLS-DA model, significantly different VOCs among the soil samples were selected by the VIP value (> 0.7) and the P value (< 0.05), as evaluated by analysis of variance for statistical significance. A total of 44 VOCs were identified (six aldehydes, six benzenoids, two sulfur-containing compounds, five alkanes and alkenes, six esters, three furans, four ketones, three terpenes, three others, and six unknowns). These possessed significantly different VOCs among various soil samples (Supplemental Table 1). The relative contents of discriminant metabolites were shown in the heat map (Fig. 2C). According to the heat map analysis, most of the benzenoids, alkanes and alkenes, ketones, and esters were relatively high in the *SMV* samples. Collectively, based on the heatmap analysis, we selected SM and *SMV* samples to understand the effect of the soil-mixing activity in human physiology.

METABOLITE ANALYSIS AND CORRELATION ANALYSIS OF SERUM METABOLITES AFTER THE EFFECT OF THE SOIL-MIXING ACTIVITY. Based on our results of soil variation with VOCs, we performed the soil-mixing activity with both the SM and *SMV*. We subjected the metabolite profiling of serum samples to identify the metabolite levels and clarify how these were affected by the soil-mixing activity. The OPLS-DA score plot for serum datasets showed a clearly distinct pattern between the control group (using SM) and treatment group (using *SMV*) (Fig. 3B). However, the PCA score plots showed an unclear cluster between experimental groups compared with OPLS-DA score plots (Fig. 3A). According to the OPLS-DA model, discriminant metabolites between the control and treatment groups were selected by the VIP value (> 1.0). A total of 59 metabolites were identified [5 organic acids, 10 amino acids, 11 carbohydrates, 17 fatty acids and lipids, 5 others, and 11 unknowns (Supplemental Table 2)]. For visualization of different metabolites, all were displayed on a heat map (Fig. 3C). Based on this, the organic acids, amino acids, and others, except for the citric acid, proline, serine, phenylalanine, tryptophan, and uric acid, were relatively higher in the treatment group than the control group. Collectively, most of the metabolites were higher in *SMV*. However, most of the fatty acids and lipids were relatively higher in the control group than in the treatment group. Furthermore, we conducted a correlation analysis between physiological measurements and significantly altered serum metabolites (Supplemental Fig. 1). Most of the amino acids, carbohydrates, and fatty acids/lipids were positively correlated with RFA and BDNF. Particularly, tryptophan and phenylalanine significantly correlated with RFA (O2) and BDNF, respectively.

Discussion

The role of soil VOCs is poorly understood, and the effects of soil-mixing activities also require further investigation. Therefore, this study investigated VOC profiling of soil and the effect of the soil-mixing activity using the metabolomic approach. The VOC analysis of various soil samples (i.e., S, SM, and *SMV*) were performed to identify the VOCs and then select the soil for the soil-mixing activity. Moreover, we analyzed the serum metabolites, EEG, and HRV between the experimental group (control vs treatment) to understand the effects of the soil-mixing activity. As a result, the effects of soil-mixing activity on human

metabolic and autonomic reactions was different, depending on the presence or absence of *M. vaccae* microorganisms in the soil.

The comparative VOC analysis of different soil samples demonstrated a marked distinction (Fig. 2). Particularly, most VOCs (including the benzenoids, sulfur-containing compounds, alkanes and alkenes, esters, and ketones) were found to be higher in *SMV* samples than in other soil samples. The effects of soil influences on human health included food production, nutrient supply, enhancement of the immune system, and as a source of medications (Brevik et al., 2020). The VOCs include aldehydes, alcohols, alkenes, benzenoids, esters, furans, ketones, sulfur-containing compounds, and terpenes, which were detected from the natural soil and forest soil (Antonelli et al., 2020; Perrault et al., 2015; Tassi et al., 2015; Wheatley et al., 1996).

Moreover, the exposure to soil VOCs has an effect on human health, such as alleviating inflammation and stress, assisting with sleep, and psychological behaviors (e.g., anxiolytic and antidepressive properties) (Antonelli et al., 2020). Furthermore, antibiotic characteristics, antimicrobial properties that were found in soil, and the exposure to soil-borne microorganisms all played a crucial role in regulation of the human immune systems (Brevik et al., 2020). Notably, exposure to *M. vaccae* in immune system activation and serotonin pathways could influence behavioral and emotional responses (Brevik et al., 2020). Some research demonstrated that injection with heat-killed *M. vaccae* to mice (*Mus musculus*) could influence immunocompetence through gastrointestinal tract interaction, immune activation of serotonergic neurons located in related parts of the brain, upregulation of serotonin metabolism in the prefrontal cortex, and the production of metabolites such as lipids (Foxx et al., 2021; Matthews and Jenks, 2013). However, a limited number of VOCs and metabolism that are derived from *M. vaccae* studies have been reported (McNerney et al., 2012; Nawrath et al., 2012). To address the gaps in previous studies, we examined the serum metabolomic difference between soil-mixing experimental groups and revealed the synergetic effect of soil-mixing activities and VOCs.

The results of the EEG showed that the RFA and ASEF50 of the right occipital lobe were significantly higher in the treatment group compared with the control group ($P < 0.05$) (Table 3). The RFA means neural oscillations in the frequency range of 11 to 13 Hz, and the ASEF50 is the frequency of the point on the power spectrum graph, in which the area from 8 to 13 Hz occupies 50% of the entire frequency range (Choi et al., 2012). Both indicators are related to alpha waves (8–13 Hz). As the size of fast frequency band among the alpha wave bands increases, the values of the two indicators appear larger. Increased cortical alpha activity was associated with states of relaxation and calmness in the brain (Basar, 2012; Iijima et al., 2009; Sayorwan et al., 2012) and was attenuated under conditions of emotional tension and stress (Kim et al., 2017; Lorig and Schwartz, 1988). In particular, an increase in the fast alpha band indicated that emotional anxiety becomes stable and that the brain is awake (Choi et al., 2012).

A previous study revealed that when 2-methylisoborneol (a major odor molecule of soil) was inhaled, the participant's fast alpha band increased significantly (Kim et al., 2017). Lorig (2000) reported that pleasant odors promote increased alpha waves, whereas unpleasant odors cause a decrease in alpha waves. It is thought that olfactory stimulation through increased soil-derived VOCs, which are caused by the addition of *M. vaccae*, made the participants feel pleasant and caused an

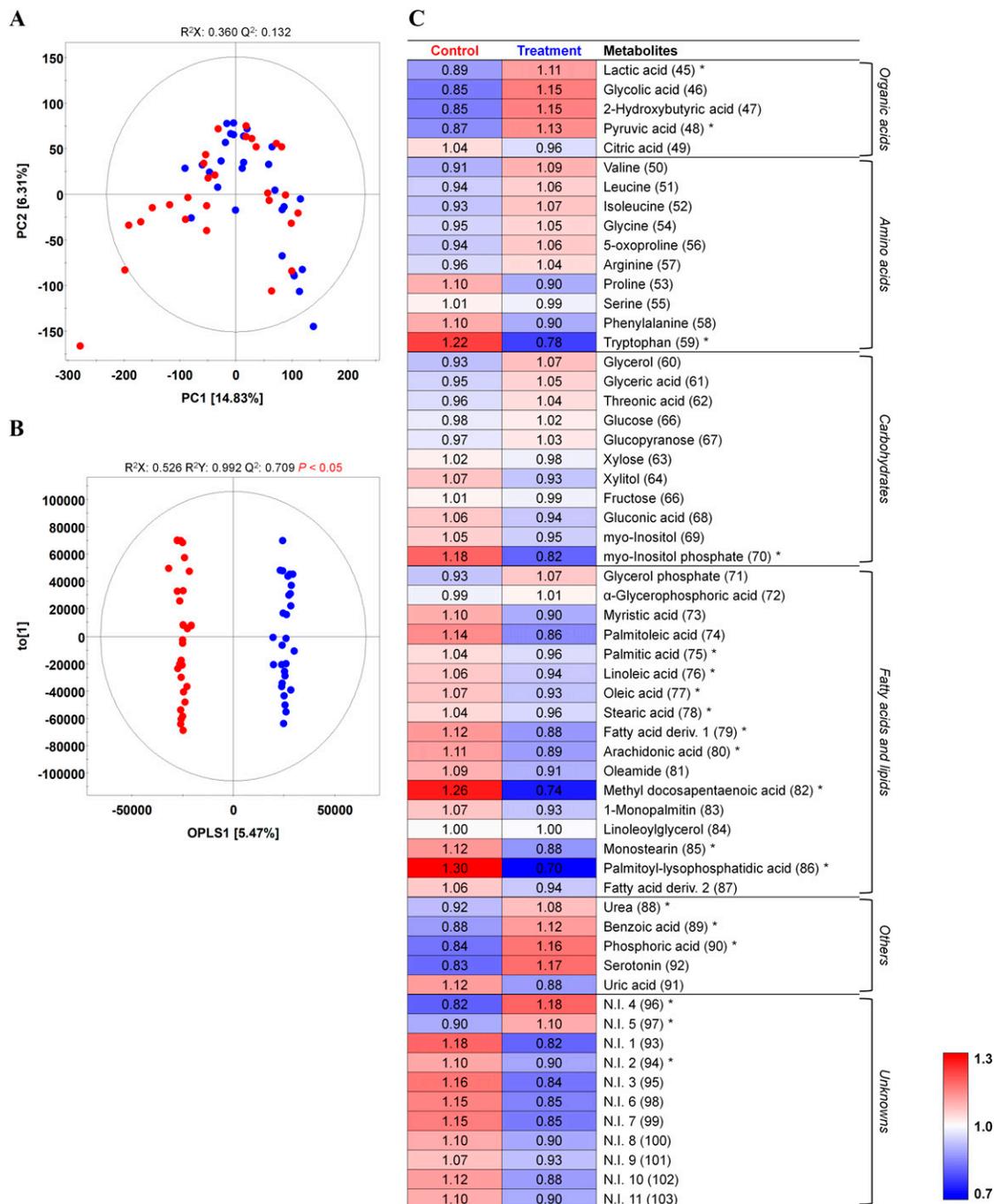


Fig. 3. (A) Principal component analysis and (B) partial least-square discriminant analysis score plot derived from the gas chromatography–time-of-flight–mass spectrometry datasets for serum samples; control [soil treated with culture media (λ)]; treatment [soil treated with *Mycobacterium vaccae* (λ)]. (C) Heat map analysis for the relative abundance of different serum metabolites (variable importance in the projection > 1.0) derived from the gas chromatography–time-of-flight–mass spectrometry analysis. The colored squares (blue to red) indicate fold changes that were normalized by the average of each metabolite. *Significantly different metabolite between control and treatment groups ($P < 0.05$, Student's t test); N.I. = nonidentified.

increase in calmness and arousal in the brain. These EEG changes may be the result of the incense component in *M. vaccae* that acts as a ligand for mammalian receptors, such as transient receptor potential vanilloid (TRPV) 3, that induce rapid changes in neurophysiology. In the previous study, insensol acetate, an incense component, was found to influence changes in emotional regulation by activating TRPV3 channels in the brain (Moussaieff et al., 2008).

The HRV measured by the ECG showed that heart rates in the treatment group were lower than in the control group [$P < 0.05$ (Table 4)]. Heart rate is controlled by the action of the autonomic nervous system, and a decreased heart rate can indicate an increase in parasympathetic activity or decrease in sympathetic outflow (Carter, 2009). Wichrowski et al. (2005) reported that when cardiac rehabilitation inpatients performed horticultural activities, the heart rate of the patients decreased significantly.

As an elevated heart rate is an indicator of a stress response (Todd, 2014), the decrease in heart rate through horticultural activity implies a stabilization of the autonomic nervous system and reduced stress levels.

Serum metabolomics showed that the treatment group possessed relatively higher levels of organic acids, amino acids, and others compared with the control group (Fig. 3). However, most of the fatty acids and lipids were relatively higher in the control group. Particularly, lactic acid was known as a crucial metabolite for brain functioning and an energy source used by neurons during activity (Todd, 2014). In addition, lactic acid optimized functioning of the gamma-aminobutyric acid receptor, ensuring that central nervous system inhibition is effectually recognized (Todd, 2014). Lactic acid could be considered vital to the maintenance of cognitive functions and protection from neuron damage (Todd, 2014). Moreover, there is limited research on the effects of pyruvic acid on cognitive functions. Research has demonstrated that pyruvate, when infused into the hippocampus and the medial septum, showed a reversal in the memory-impairing effects of morphine or septal muscimol, a GABA_A receptor agonist (Owen and Sunram-Lea, 2011).

Tryptophan is an essential amino acid, derived from various protein-based foods and dietary proteins. It is the precursor of various physiologically essential metabolites, such as kynurenine and serotonin (Jenkins et al., 2016). Serotonin has been correlated with motivational and emotional aspects of human behavior, such as depression, anxiety disorders, and compulsive disorders (Silber and Schmitt, 2010). Psychological disorders of depression and emotions such as loneliness have been correlated with serotonin levels, which showed decreasing patterns (Park et al., 2020). Furthermore, serotonin-related drugs have been commonly used in the treatment of psychological abnormalities (Meneses and Liy-Salmeron, 2012). Notably, most fatty acids and lipids showed relatively lower levels in treatment groups (Fig. 3). An excessive level of fat in the diet might influence neuropsychiatric functions negatively, through cognition, anxiety, and emotional levels (Moon et al., 2014). Elevated contents of palmitic and linoleic acid were negatively linked to anxiety-like behavior and cognitive functions (Bernard et al., 2015; Moon et al., 2014). Moreover, elevation of these metabolites in plasma were correlated with diseases such as metabolic syndrome, obesity, and poor clinical outcomes (Moon et al., 2014). The decreased fatty acids, such as palmitic acid in the *SMV* group, could be due to metabolism of lipids by *M. vaccae*. Previous studies have shown that mycobacteria synthesize *cis*-10-hexadecenoic acid from palmitic acid (Scheuerbrandt and Bloch, 1962), which in turn binds to the host peroxisome proliferator-activated receptor-alpha receptor and has been shown to have anti-inflammatory effects (Smith et al., 2019).

In this study, the levels of organic acids (lactic and pyruvic acids and serotonin) were shown to increase, whereas most fatty acids and lipids (including palmitic and linoleic acid) decreased in soil-mixing activities inoculated by the *M. vaccae* strain. These results can be beneficial in explaining the effect of *M. vaccae* soil-mixing activities on physiological and psychiatric disorders. Furthermore, the correlation analysis showed that tryptophan and serotonin were positively correlated with RFA (O₂), ASEF50 (O₂), and BDNF (Supplemental Fig. 2). These results were similar to our previous research on gardening intervention (Park et al., 2020). Components of incenses could potentially act not only on the primary olfactory cortex but also on the mesocorticolimbic

dopaminergic and serotonergic systems (Iijima et al., 2009). In particular, several 5-hydroxytryptamine (serotonin) receptors were expressed highly by most excitatory neurons in the occipital cortex (Beliveau et al., 2017; Watakabe et al., 2009). Therefore, this positive correlation may have appeared as an association between the activity of the occipital cortex and the activity of the serotonin system.

In conclusion, the soil-mixing activity with *M. vaccae* showed an increased level of organic acids (including lactic and pyruvic acids and serotonin) and decreased levels of fatty acids and lipids (including palmitic and linoleic acids). This could contribute to improvements in psychological health. In addition, the alpha wave of the occipital cortex increased, and the heart rate decreased, resulting in a stabilizing effect on the autonomic nervous system. This study suggests that human contact with and exposure to soil and microbes correlates with human health for both psychological and therapeutic aspects. This physiological response appeared immediately, and it could be predicted that this is because the *M. vaccae* was introduced through the oral cavity and upper respiratory tract, which are the main ports for microorganisms to enter the human body (Macovei et al., 2015).

The main limitations of this study are the small sample size and lack of representativeness of the sample because the participants were mostly healthy women. However, this study is valuable because it provided new data that could fill the void presented in previous studies by measuring the effects of soil-mixing activity on human psycho-physiological aspects, depending on the presence or absence of soil microorganisms. This study contributes to propose the role of soil microorganisms in the therapeutic effects of contact with soil and humans.

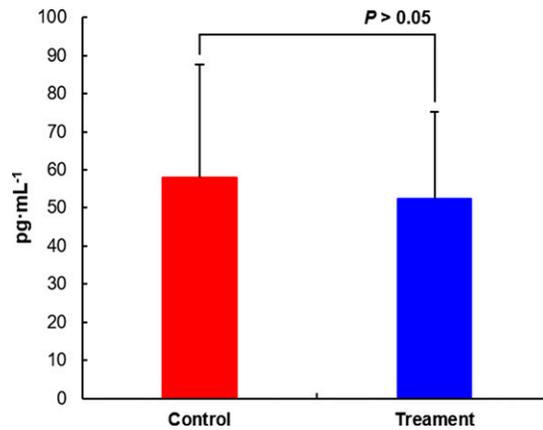
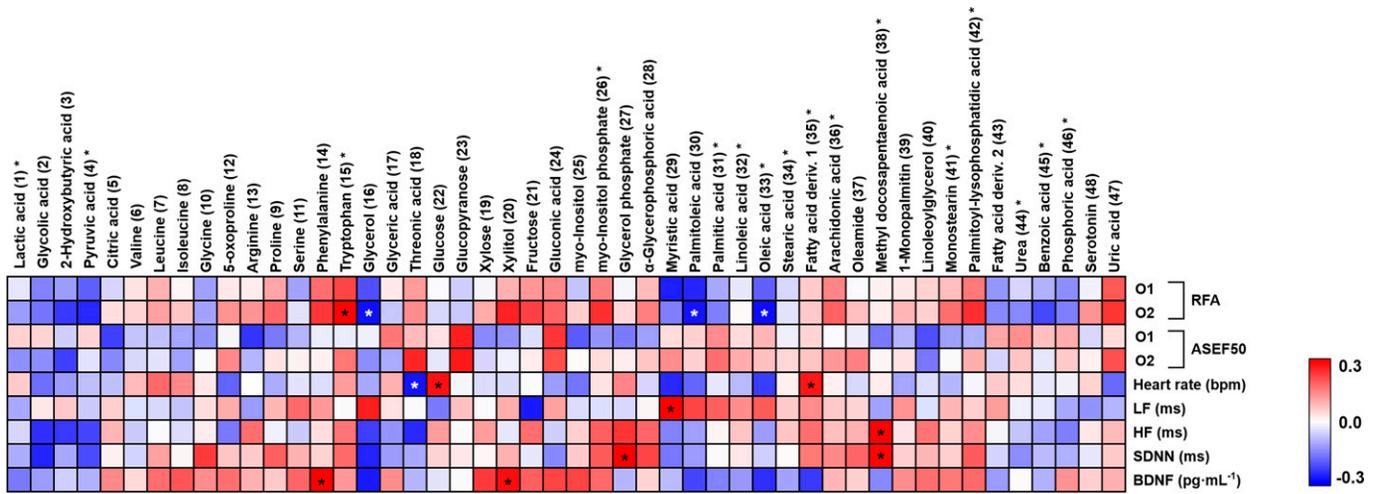
In the future, the impact of various soil microorganisms on human physiological health should be explored. In addition, further studies should be conducted to develop soil with therapeutic functions for relevant participants, such as older adults and people with disabilities, and to develop long-term programs using soil for maintaining and promoting the health of healthy participants.

Literature Cited

- Antonelli, M., D. Donelli, G. Barbieri, M. Valussi, V. Maggini, and F. Firenzuoli. 2020. Forest volatile organic compounds and their effects on human health: A state-of-the-art review. *Int. J. Environ. Res. Public Health* 17:6506, <https://doi.org/10.3390/ijerph17186506>.
- Basar, E. 2012. A review of alpha activity in integrative brain function: Fundamental physiology, sensory coding, cognition and pathology. *Int. J. Psychophysiol.* 86:1–24, <https://doi.org/10.1016/j.jpsycho.2012.07.002>.
- Beliveau, V., M. Ganz, L. Feng, B. Ozenne, L. Højgaard, P.M. Fisher, C. Svarer, D.N. Greve, and G.M. Knudsen. 2017. A high-resolution in vivo atlas of the human brain's serotonin system. *J. Neurosci.* 37:120–128, <https://doi.org/10.1523/JNEUROSCI.2830-16.2016>.
- Bernard, J.Y., M. Armand, C. Garcia, A. Forhan, M. De Agostini, M.A. Charles, and B. Heude. 2015. The association between linoleic acid levels in colostrum and child cognition at 2 and 3 y in the EDEN cohort. *Pediatr. Res.* 77:829–835, <https://doi.org/10.1038/pr.2015.50>.
- Brevik, E.C., L. Slaughter, B.R. Singh, J.J. Steffan, D. Collier, P. Barnhart, and P. Pereira. 2020. Soil and human health: Current status and future needs. *Air Soil Water Res.* 13:1–23, <https://doi.org/10.1177/1178622120934441>.
- Carter, R. 2009. *The human brain book: An illustrated guide to its structure, function, and disorders*. Penguin, London, UK.
- Choi, S.W., H.H. Jo, J.W. Heo, K.S. Kim, and S.Y. Jeong. 2012. *Introduction to neurofeedback*. Sihmapress, Seoul, Republic of Korea.

- Dye, C. 2008. Health and urban living. *Science* 319:766–769, <https://doi.org/10.1126/science.1150198>.
- Fonken, L.K., M.G. Frank, H.M. D’Angelo, J.D. Heinze, L.R. Watkins, C.A. Lowry, and S.F. Maier. 2018. *Mycobacterium vaccae* immunization protects aged rats from surgery-elicited neuroinflammation and cognitive dysfunction. *Neurobiol. Aging* 71:105–114, <https://doi.org/10.1016/j.neurobiolaging.2018.07.012>.
- Fox, C.L., J.D. Heinze, A. González, F. Vargas, M.V. Baratta, A.I. Elsayed, J.R. Stewart, K.M. Loupy, M.R. Arnold, M.C. Flux, S.A. Sago, P.H. Siebler, L.N. Milton, M.W. Lieb, J.E. Hassell, D.G. Smith, K.A.K. Lee, S.A. Appiah, E.J. Schaefer, M. Panitchpakdi, N.C. Sikora, K.C. Weldon, C.E. Stamper, D. Schmidt, D.A. Duggan, Y.M. Mengesha, M. Ogbaslassie, K.T. Nguyen, C.A. Gates, K. Schnabel, L. Tran, J.D. Jones, M.H. Vitaterna, F.W. Turek, M. Fleshner, P.C. Dorrestein, R. Knight, K.P. Wright, and C.A. Lowry. 2021. Effects of immunization with the soil-derived bacterium *Mycobacterium vaccae* on stress coping behaviors and cognitive performance in a “two hit” stressor model. *Front. Physiol.* 11:524833, <https://doi.org/10.3389/fphys.2020.524833>.
- Frank, M.G., L.K. Fonken, S.D. Dolzani, J.L. Annis, P.H. Siebler, D. Schmidt, L.R. Watkins, S.F. Maier, and C.A. Lowry. 2018. Immunization with *Mycobacterium vaccae* induces an anti-inflammatory milieu in the CNS: Attenuation of stress-induced microglial priming, alarmins and anxiety-like behavior. *Brain Behav. Immun.* 73:352–363, <https://doi.org/10.1016/j.bbi.2018.05.020>.
- Hoisington, A.J., L.A. Brenner, K.A. Kinney, T.T. Postolache, and C.A. Lowry. 2015. The microbiome of the built environment and mental health. *Microbiome* 3:60, <https://doi.org/10.1186/s40168-015-0127-0>.
- Human Metabolome Database. 2021. Browse metabolites. 12 July 2021. <<http://www.hmdb.ca/metabolites>>.
- Iijima, M., M. Osawa, N. Nishitani, and M. Iwata. 2009. Effects of incense on brain function: Evaluation using electroencephalograms and event-related potentials. *Neuropsychobiol.* 59:80–86, <https://doi.org/10.1159/000209859>.
- Ikei, H., M. Komatsu, C. Song, E. Himoro, and Y. Miyazaki. 2014. The physiological and psychological relaxing effects of viewing rose flowers in office workers. *J. Physiol. Anthropol.* 33:6, <https://doi.org/10.1186/1880-6805-33-6>.
- Jasper, H.H. 1958. The ten-twenty electrode system of the International Federation. *Electroencephalogr. Clin. Neurophysiol.* 10:370–375.
- Jenkins, T.A., J.C. Nguyen, K.E. Polglaze, and P.P. Bertrand. 2016. Influence of tryptophan and serotonin on mood and cognition with a possible role of the gut-brain axis. *Nutrients* 8:56, <https://doi.org/10.3390/nu8010056>.
- Kim, M., K. Sowndhararajan, T. Kim, J.E. Kim, J.E. Yang, and S. Kim. 2017. Gender differences in electroencephalographic activity in response to the earthy odorants geosmin and 2-methylisoborneol. *Appl. Sci. (Basel)* 7:876, <https://doi.org/10.3390/app7090876>.
- Kim, S.O., Y.A. Oh, and S.A. Park. 2020. Foliage plants improve concentration and emotional condition of elementary school students performing an intensive assignment. *HortScience* 55:378–385, <https://doi.org/10.21273/HORTSCI14757-19>.
- Krabbendam, L. and J. van Os. 2005. Schizophrenia and urbanicity: A major environmental influence – Conditional on genetic risk. *Schizophr. Bull.* 31:795–799, <https://doi.org/10.1093/schbul/sbi060>.
- Lorig, T.S. 1989. Human EEG and odor response. *Prog. Neurobiol.* 33:387–398, [https://doi.org/10.1016/0301-0082\(89\)90007-5](https://doi.org/10.1016/0301-0082(89)90007-5).
- Lorig, T.S. 2000. The application of electroencephalographic techniques to the study of human olfaction: A review and tutorial. *Int. J. Psychophysiol.* 36:91–104, [https://doi.org/10.1016/S0167-8760\(99\)00104-X](https://doi.org/10.1016/S0167-8760(99)00104-X).
- Lorig, T.S. and G.E. Schwartz. 1988. Brain and odor: I. Alteration of human EEG by odor administration. *Psychobiol.* 16:281–284, <https://doi.org/10.3758/BF03327318>.
- Lowry, C.A., J.H. Hollis, A. De Vries, B. Pan, L.R. Brunet, J.R. Hunt, J.F.R. Paton, E.V. Kampen, D.M. Knight, A.K. Evans, G.A. Rook, and S.L. Lightman. 2007. Identification of an immune-responsive mesolimbocortical serotonergic system: Potential role in regulation of emotional behavior. *Neuroscience* 146:756–772, <https://doi.org/10.1016/j.neuroscience.2007.01.067>.
- Macovei, L., J. McCafferty, T. Chen, F. Teles, H. Hasturk, B.J. Paster, and A. Campos-Neto. 2015. The hidden ‘mycobacteriome’ of the human healthy oral cavity and upper respiratory tract. *J. Oral Microbiol.* 7:26094, <https://doi.org/10.3402/jom.v7.26094>.
- Masago, R., T. Matsuda, Y. Kikuchi, Y. Miyazaki, K. Iwanaga, H. Harada, and T. Katsuura. 2000. Effects of inhalation of essential oils on EEG activity and sensory evaluation. *J. Physiol. Anthropol. Appl. Human Sci.* 19:35–42, <https://doi.org/10.2114/jpa.19.35>.
- Mathews, D.M. and S.M. Jenks. 2013. Ingestion of *Mycobacterium vaccae* decreases anxiety-related behavior and improves learning in mice. *Behav. Processes* 96:27–35, <https://doi.org/10.1016/j.beproc.2013.02.007>.
- McNerney, R., K. Mallard, P.L. Okolo, and C. Turner. 2012. Production of volatile organic compounds by mycobacteria. *FEMS Microbiol. Lett.* 328:150–156, <https://doi.org/10.1111/j.1574-6968.2011.02493.x>.
- Meneses, A. and G. Liy-Salmeron. 2012. Serotonin and emotion, learning and memory. *Rev. Neurosci.* 23:543–553, <https://doi.org/10.1515/revneuro-2012-0060>.
- Moon, M.L., J.J. Joesting, M.A. Lawson, G.S. Chiu, N.A. Blevins, K.A. Kwakwa, and G.G. Freund. 2014. The saturated fatty acid, palmitic acid, induces anxiety-like behavior in mice. *Metabolism* 63:1131–1140, <https://doi.org/10.1016/j.metabol.2014.06.002>.
- Moussaieff, A., N. Rimmerman, T. Bregman, A. Straiker, C.C. Felder, S. Shoham, Y. Kashman, S.M. Huang, H. Lee, E. Shohami, K. Mackie, M.J. Caterina, J.M. Walker, E. Fride, and R. Mechoulam. 2008. Incensole acetate, an incense component, elicits psychoactivity by activating TRPV3 channels in the brain. *FASEB J.* 22:3024–3034, <https://doi.org/10.1096/fj.07-101865>.
- Nawrath, T., G.F. Mgode, B. Weetjens, S.H. Kaufmann, and S. Schulz. 2012. The volatiles of pathogenic and nonpathogenic mycobacteria and related bacteria. *Beilstein J. Org. Chem.* 8:290–299, <https://doi.org/10.3762/bjoc.8.31>.
- O’Brien, M.E.R., H. Anderson, E. Kaukel, K. O’Byrne, M. Pawlicki, J. Von Pawel, and M. Reck. 2004. SRL172 (killed *Mycobacterium vaccae*) in addition to standard chemotherapy improves quality of life without affecting survival, in patients with advanced non-small-cell lung cancer: Phase III results. *Ann. Oncol.* 15:906–914, <https://doi.org/10.1093/annonc/mdh220>.
- Orians, G.H. 1986. An ecological and evolutionary approach to landscape aesthetics. Routledge, London, UK.
- Owen, L. and S.I. Sunram-Lea. 2011. Metabolic agents that enhance ATP can improve cognitive functioning: A review of the evidence for glucose, oxygen, pyruvate, creatine, and L-carnitine. *Nutrients* 3:735–755, <https://doi.org/10.3390/nu3080735>.
- Park, S.A., C. Song, J.Y. Choi, K.C. Son, and Y. Miyazaki. 2016. Foliage plants cause physiological and psychological relaxation as evidenced by measurements of prefrontal cortex activity and profile of mood states. *HortScience* 51:1308–1312, <https://doi.org/10.21273/HORTSCI11104-16>.
- Park, S.A., C. Song, Y.A. Oh, Y. Miyazaki, and K.C. Son. 2017. Comparison of physiological and psychological relaxation using measurements of heart rate variability, prefrontal cortex activity, and subjective indexes after completing tasks with and without foliage plants. *Int. J. Environ. Res. Public Health* 14:1087, <https://doi.org/10.3390/ijerph14091087>.
- Park, S.A., S.Y. Son, A.Y. Lee, H.G. Park, W.L. Lee, and C.H. Lee. 2020. Metabolite profiling revealed that a gardening activity program improves cognitive ability correlated with BDNF levels and serotonin metabolism in the elderly. *Int. J. Environ. Res. Public Health* 17:541, <https://doi.org/10.3390/ijerph17020541>.
- Pedersen, C.B. and P.B. Mortensen. 2001. Evidence of a dose-response relationship between urbanicity during upbringing and schizophrenia risk. *Arch. Gen. Psychiatry* 58:1039–1046, <https://doi.org/10.1001/archpsyc.58.11.1039>.

- Peen, J., R.A. Schoevers, A.T. Beekman, and J. Dekker. 2010. The current status of urban-rural differences in psychiatric disorders. *Acta Psychiatr. Scand.* 121:84–93, <https://doi.org/10.1111/j.1600-0447.2009.01438.x>.
- Perrault, K.A., P.H. Stefanuto, B.H. Stuart, T. Rai, J.F. Focant, and S.L. Forbes. 2015. Detection of decomposition volatile organic compounds in soil following removal of remains from a surface deposition site. *Forensic Sci. Med. Pathol.* 11:376–387, <https://doi.org/10.1007/s12024-015-9693-5>.
- Reber, S.O., P.H. Siebler, N.C. Donner, J.T. Morton, D.G. Smith, J.M. Kopelman, K.R. Lowe, K.J. Wheeler, J.H. Fox, J.E. Hassell, Jr., B.N. Greenwood, C. Jansch, A. Lechner, D. Schmidt, N. Uschold-Schmidt, A.M. Füchsl, D. Langgartner, F.R. Walker, M.W. Hale, G.L. Perez, W.V. Treuren, A. González, A.L. Halweg-Edwards, M. Fleshner, C.L. Raison, G.A. Rook, S.D. Peddada, R. Knight, and C.A. Lowry. 2016. Immunization with a heat-killed preparation of the environmental bacterium *Mycobacterium vaccae* promotes stress resilience in mice. *Proc. Natl. Acad. Sci. USA* 113:E3130–E3139, <https://doi.org/10.1073/pnas.1600324113>.
- Rook, G.A., V. Adams, J. Hunt, R. Palmer, R. Martinelli, and L.R. Brunet. 2004. Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. *Springer Semin. Immun.* 25:237–255, <https://doi.org/10.1007/s00281-003-0148-9>.
- Roslund, M.I., R. Puhakka, M. Grönroos, N. Nurminen, S. Oikarinen, A.M. Gazali, O. Cinek, L. Kramna, N. Siter, H.K. Vari, L. Soininen, A. Parajuli, J. Rajaniemi, T. Kinnunen, O.H. Laitinen, H. Hyoty, A. Sinkkonen, and ADELE research group. 2020. Biodiversity intervention enhances immune regulation and health-associated commensal microbiota among daycare children. *Sci. Adv.* 6:eaba2578, <https://doi.org/10.1126/sciadv.aba2578>.
- Roslund, M.I., R. Puhakka, N. Nurminen, S. Oikarinen, N. Siter, M. Grönroos, O. Cinek, L. Kramna, A. Jumpponen, O.H. Laitinen, J. Rajaniemi, H. Hyoty, A. Sinkkonen, and ADELE research group. 2021. Long-term biodiversity intervention shapes health-associated commensal microbiota among urban day-care children. *Environ. Int.* 157:106811, <https://doi.org/10.1016/j.envint.2021.106811>.
- Sayorwan, W., V. Siripompanich, T. Piriyaunyaporn, T. Hongratana-worakit, N. Kotchabhakdi, and N. Ruangrunsi. 2012. The effects of lavender oil inhalation on emotional states, autonomic nervous system, and brain electrical activity. *J. Med. Assoc. Thai.* 95:598–606.
- Scheuerbrandt, G. and K. Bloch. 1962. Unsaturated fatty acids in microorganisms. *J. Biol. Chem.* 237:2064–2068, [https://doi.org/10.1016/S0021-9258\(19\)63397-1](https://doi.org/10.1016/S0021-9258(19)63397-1).
- Silber, B.Y. and J.A.J. Schmitt. 2010. Effects of tryptophan loading on human cognition, mood, and sleep. *Neurosci. Biobehav. Rev.* 34: 387–407, <https://doi.org/10.1016/j.neubiorev.2009.08.005>.
- Singh, D. and C.H. Lee. 2018. Intraspecies volatile interactions affect growth rates and exometabolomes in *Aspergillus oryzae* KCCM 60345. *J. Microbiol. Biotechnol.* 28:199–209, <https://doi.org/10.4014/jmb.1711.11005>.
- Smith, D.G., R. Martinelli, G.S. Besra, P.A. Illarionov, I. Szatmari, P. Brazda, M.A. Allen, W. Xu, W. Wang, L. Nagy, R.D. Dowell, G.A.W. Rook, L.R. Brunet, and C.A. Lowry. 2019. Identification and characterization of a novel anti-inflammatory lipid isolated from *Mycobacterium vaccae*, a soil-derived bacterium with immunoregulatory and stress resilience properties. *Psychopharmacology (Berl.)* 236:1653–1670, <https://doi.org/10.1007/s00213-019-05253-9>.
- Sowndhararajan, K., H. Cho, B. Yu, and S. Kim. 2015. Effect of olfactory stimulation of isomeric aroma compounds, (+)-limonene and terpinolene on human electroencephalographic activity. *Eur. J. Integr. Med.* 7:561–566, <https://doi.org/10.1016/j.eujim.2015.08.006>.
- Tassi, F., S. Venturi, J. Cabassi, F. Capecciacci, B. Nisi, and O. Vaselli. 2015. Volatile organic compounds (VOCs) in soil gases from Solfatara crater (Campi Flegrei, southern Italy): Geogenic source (s) vs. biogeochemical processes. *Appl. Geochem.* 56:37–49, <https://doi.org/10.1016/j.apgeochem.2015.02.005>.
- Todd, J.J. 2014. Lactate: Valuable for physical performance and maintenance of brain function during exercise. *Biosci. Horizons Int. J. Student Res.* 7: <https://doi.org/10.1093/biohorizons/hzu001>.
- UKessays. 2020. Occipital lobe: Function in the brain. 4 Aug. 2021. <<https://www.ukessays.com/essays/biology/analysis-of-the-occipital-lobe-in-human-brain.php?vref=1>>.
- Ulrich, R.S. 1983. Aesthetic and affective response to natural environment, p. 85–125. In: I. Altman and J.F. Wohlwill. *Behavior and the natural environment*. Springer, Cham, Switzerland. https://doi.org/10.1007/978-1-4613-3539-9_4.
- Watakabe, A., Y. Komatsu, O. Sadakane, S. Shimegi, T. Takahata, N. Higo, S. Tochitani, T. Hashikawa, T. Naito, H. Osaki, H. Sakamoto, M. Okamoto, A. Ishikawa, S.I. Hara, T. Akasaki, H. Sato, and T. Yamamori. 2009. Enriched expression of serotonin 1B and 2A receptor genes in macaque visual cortex and their bidirectional modulatory effects on neuronal responses. *Cereb. Cortex* 19:1915–1928, <https://doi.org/10.1093/cercor/bhn219>.
- Wheatley, R.E., S.E. Millar, and D.W. Griffiths. 1996. The production of volatile organic compounds during nitrogen transformations in soils. *Plant Soil* 181:163–167, <https://doi.org/10.1007/BF00011303>.
- Wichrowski, M., J. Whiteson, F. Haas, A. Mola, and M.J. Rey. 2005. Effects of horticultural therapy on mood and heart rate in patients participating in an inpatient cardiopulmonary rehabilitation program. *J. Cardiopulm. Rehabil.* 25:270–274, <https://doi.org/10.1097/00008483-200509000-00008>.
- Wilson, E.O. 1984. *Biophilia*. Harvard Univ. Press, Cambridge, MA.



Supplemental Table 1. Significantly different volatile organic compounds identified by solid-phase microextraction gas chromatography–time-of-flight–mass spectrometry in soil sample treated distilled water and culture media and inoculated *Mycobacterium vaccae*.

No.	Ret (min) ^z	VIP1 ^y	VIP2	Tentative identification ^x	Unique mass (m/z)	MS fragment pattern (m/z)	P value	ID ^w
Aldehydes								
1	4.27	0.19	1.41	2-Methyl-2-butenal	55	55, 84, 53, 50, 51, 56, 83, 79, 85	0.000	MS ^v
2	5.46	1.41	1.04	Hexanal	56	56, 57, 60, 55, 72, 67, 73	0.000	MS
3	7.57	1.45	1.03	Heptanal	55	55, 70, 57, 81, 71, 68	0.000	MS
4	8.71	0.09	1.41	Benzaldehyde	105	77, 51, 105, 106, 50, 78	0.000	MS
5	9.46	1.44	1.04	Octanal	57	57, 55, 56, 69, 84	0.000	MS
6	10.21	0.54	1.35	Benzene acetaldehyde	91	91, 65, 92, 63, 120	0.000	MS
Benzenoids								
7	4.74	0.69	1.21	Methylbenzene	91	91, 92, 65, 63, 51, 50	0.009	MS
8	9.31	0.96	0.97	Mesitylene (Benzene, 1,3,5-trimethyl-)	105	55, 69, 105, 70, 56, 57, 53	0.047	MS
9	9.86	0.93	1.17	o-Cymene	119	119, 91, 117, 65, 77, 134	0.005	MS
10	11.37	1.18	1.09	Benzene ethanol	92	91, 92, 65, 51, 122, 63	0.003	MS
11	12.38	1.26	1.17	Benzeneacetic acid, methyl ester	91	91, 65, 150, 63, 59, 89	0.000	MS
12	13.48	1.23	1.2	Benzene, 1,3-bis(1,1-dimethylethyl)-	175	57, 175, 91, 65, 115	0.000	MS
Sulfur-containing compounds								
13	4.28	1.42	1.09	Dimethyl disulfide	94	94, 79, 61, 64, 96, 81	0.000	MS
14	8.85	1.29	1.14	Dimethyl Trisulfide	79	79, 126, 64, 78, 80	0.000	MS
Alkanes and alkenes								
15	5.04	1.22	1.2	2,2,4-Trimethylhexane	57	57, 56, 71, 55, 58, 70	0.000	MS
16	6.32	1.21	1.2	2,4-Dimethyl-1-heptene	55	55, 70, 57, 56, 69, 83	0.000	MS
17	7.06	1.26	1.18	2,2-Dimethylheptane	57	57, 56, 71, 55, 58, 51, 77	0.000	MS
18	8.96	0.88	1.24	Cyclohexane, 1-methyl-4-(1-methylethyl)-, trans	97	55, 97, 81, 96, 69	0.001	MS
19	13.06	1.46	1.04	5-Methyl-1-undecene	57	56, 57, 55, 70	0.000	MS
Esters								
20	4.99	1.09	1.01	Methyl-3-methylbutanoate	74	74, 59, 57, 85, 101, 69	0.018	MS
21	5.81	1.16	1.19	Methyl-3,3-dimethylbutanoate	73	73, 74, 57, 55, 59, 53, 99	0.000	MS
22	6.52	1.26	1.16	Methyl-2,3-dimethylbutanoate	88	88, 57, 59, 55, 56, 71, 87	0.000	MS
23	7.98	1.15	1.19	Methyl-2-ethylpentanoate	87	87, 102, 55, 59, 69	0.000	MS
24	10.16	1.26	1.18	Methyl-2-ethylhexanoate	87	87, 57, 102, 55, 53, 59	0.000	MS
25	10.32	1.28	1.17	Methyl-6-methyl heptanoate	74	74, 55, 59, 87, 69	0.000	MS
Furans								
26	6.18	1.47	1.05	Fufural	96	95, 96, 67, 50, 51, 97	0.000	MS
27	8.77	1.44	1.05	5-Methylfurfural	110	53, 110, 109, 51, 50, 81	0.000	MS
28	11.3	0.96	1.06	2-Methylbenzofuran	131	131, 132, 51, 77, 50, 78	0.021	MS
Ketones								
29	6.65	1.29	1.15	6-Methyl-3,5-heptadiene-2-one	109	109, 79, 81, 124, 53, 51	0.000	MS
30	8.29	0.31	1.42	4-Methyl-2-heptanone	58	58, 55, 59, 85, 70	0.000	MS
31	8.96	1.24	1.15	1-(2-Furanyl)-2-hydroxy ethanone	95	95, 126, 67, 96, 68	0.000	MS
32	9.25	1.47	1.04	2-Octanone	58	58, 59, 71, 85, 57, 72	0.000	MS
Terpenes								
33	8.48	0.93	1.28	Camphene	93	93, 79, 91, 77, 67, 55, 121	0.000	MS
34	12.23	1.16	1.15	endo-Borneol	95	95, 55, 67, 77, 53, 69	0.001	MS
35	15.7	1.21	1.15	Aromandendrene	91	91, 79, 105, 93, 77	0.000	MS
Others								
36	7.7	1.31	0.98	Oxime-, methoxy-phenyl-	133	133, 151, 135, 77, 68, 134	0.007	MS
37	7.7	1.25	1.12	2,5-Dimethylpyrazine	108	108, 81, 52, 51, 80, 53	0.000	MS
38	9.11	0.45	1.4	Hexanoic acid	60	60, 73, 55, 57, 87, 61	0.000	MS

(Continued on next page)

Supplemental Table 1. (Continued)

No.	Ret (min) ^z	VIP1 ^y	VIP2	Tentative identification ^x	Unique mass (<i>m/z</i>)	MS fragment pattern (<i>m/z</i>)	<i>P</i> value	ID ^w
Unknown								
39	8.48	1.15	1.23	N.I. ^u 1	68	55, 68, 56, 69, 112, 84	0.000	MS
40	8.85	1.26	1.15	N.I. 2	74	74, 87, 59, 55, 69, 71	0.000	MS
41	10.25	0.57	1.31	N.I. 3	58	58, 71, 69, 84, 55	0.002	MS
42	12.82	1.44	1.03	N.I. 4	57	57, 71, 56, 55, 69	0.000	MS
43	14.27	1.27	1.17	N.I. 5	88	88, 79, 94, 57, 67, 95, 55, 77	0.000	MS
44	15.48	1.46	1.03	N.I. 6	58	58, 55, 71, 59, 57, 73	0.000	MS

^zRetention time.^yVariable importance in the projection (VIP).^xVolatile organic compounds selected by VIP (>0.7) based on partial least squares-discriminant analysis model and *P* < 0.05.^wIdentification.^vMass spectrum comparison with the Human Metabolome Database (2021), the National Institute of Standards and Technology database (version 2.0, 2011; FairCom, Gaithersburg, MD), and Wiley 9 database (Wiley-VCH, Weinheim, Germany).^uNonidentified.

Supplemental Table 2. Significantly different serum metabolites between soil-mixing activity group including control and treatment participants analyzed gas chromatography–time-of-flight–mass spectrometry analysis.

No.	Ret (min) ^z	VIP1 ^y	Unique mass (m/z)	Tentative identification ^x	MS fragment pattern (m/z)	ID ^w	P value
Organic acids							
45	5.26	26.46	117	Lactic acid	73, 147, 117, 191, 148, 75, 190, 66	STD/MS ^v	0.012
46	5.4	1.27	205	Glycolic acid	73, 147, 66, 148, 205, 75, 58, 177, 133	MS	0.109
47	5.97	3.03	148	2-Hydroxybutyric acid	73, 147, 131, 75, 58, 148, 71, 79, 66, 133	MS	0.091
48	6.13	15.45	133	Pyruvic acid	73, 147, 133, 59, 235, 220, 148, 146	STD/MS	0.005
49	12.03	1.41	273	Citric acid	73, 147, 273, 375, 347, 363, 148, 133	STD/MS	0.573
Amino acids							
50	6.91	12.28	144	Valine	144, 73, 218, 147, 145, 100, 146, 219	STD/MS	0.104
51	7.46	5.26	158	Leucine	73, 158, 147, 117, 103, 186, 133, 59	STD/MS	0.347
52	7.68	5.06	158	Isoleucine	73, 158, 181, 75, 147, 159, 100, 174	STD/MS	0.259
53	7.75	7.37	142	Proline	73, 174, 142, 147, 86, 248, 100, 133	STD/MS	0.262
54	7.82	2.93	174	Glycine	73, 174, 147, 86, 175, 248, 100, 75	STD/MS	0.292
55	8.31	1.04	204	Serine	73, 188, 100, 147, 204, 189, 59, 218	STD/MS	0.787
56	9.78	18.57	156	5-oxoproline	156, 73, 147, 75, 230, 258, 158, 148, 133	STD/MS	0.140
57	10.25	3.59	142	Arginine	73, 142, 186, 147, 216, 143, 288, 187, 133	STD/MS	0.387
58	10.62	6.01	218	Phenylalanine	73, 218, 192, 147, 75, 100, 219, 193, 117	STD/MS	0.204
59	14.61	15.01	202	Tryptophan	202, 73, 117, 291, 55, 204, 147, 218	STD/MS	0.006
Carbohydrates							
60	7.48	6.37	205	Glycerol	73, 147, 205, 117, 103, 158, 186, 133	STD/MS	0.129
61	8.04	4.08	189	Glyceric acid	73, 147, 189, 292, 103, 133, 205, 117	STD/MS	0.188
62	10.07	2.35	292	Threonic acid	73, 147, 292, 205, 117, 220, 103, 217	STD/MS	0.256
63	10.82	2.64	204	Xylose	73, 147, 204, 205, 217, 117, 103, 129	STD/MS	0.734
64	11.34	1.52	103	Xylitol	73, 147, 103, 217, 207, 205, 117, 129, 307	STD/MS	0.327
65	12.46	3.67	103	Fructose	73, 103, 217, 307, 147, 133, 308, 218	STD/MS	0.539
66	12.66	6.74	205	Glucose	73, 205, 319, 147, 160, 103, 320, 217, 117	STD/MS	0.359
67	13.14	1.82	204	Glucopyranose	73, 204, 191, 147, 205, 217, 206, 129, 133	MS	0.692
68	13.35	1.77	333	Gluconic acid	73, 147, 117, 217, 205, 333, 103, 292, 129	STD/MS	0.151
69	13.91	1.22	217	myo-Inositol	73, 147, 217, 305, 191, 368, 133, 318	STD/MS	0.459
70	15.85	1.95	318	myo-Inositol phosphate	73, 58, 147, 55, 103, 117, 217, 318, 299	MS	0.012
Fatty acids and lipids							
71	11.37	1.25	299	Glycerol phosphate	73, 147, 243, 207, 299, 129, 103, 211, 133	MS	0.081
72	11.63	16.02	357	α-Glycerophosphoric acid	73, 299, 357, 147, 103, 75, 300, 129, 315	MS	0.848
73	12.12	1.08	285	Myristic acid	73, 117, 103, 285, 132, 129, 147, 217	STD/MS	0.303
74	13.31	1.74	311	Palmitoleic acid	73, 117, 129, 55, 145, 132, 311, 147	STD/MS	0.300
75	13.43	8.96	313	Palmitic acid	73, 117, 313, 132, 129, 145, 131, 133	STD/MS	0.006
76	14.47	6.58	337	Linoleic acid	73, 117, 55, 129, 339, 145, 67, 81, 96, 132	STD/MS	0.001
77	14.49	5.99	339	Oleic acid	73, 117, 55, 129, 339, 67, 132, 84, 131	STD/MS	0.050
78	14.62	7.66	341	Stearic acid	73, 117, 132, 341, 129, 145, 131, 342, 133	STD/MS	0.005
79	14.98	1.69	337	Fatty acid deriv. 1	73, 55, 67, 207, 117, 81, 79, 129, 147, 93	MS	0.000
80	15.39	7.00	91	Arachidonic acid	73, 75, 79, 67, 55, 80, 91, 93, 117, 129	STD/MS	0.001
81	15.64	10.49	131	Oleamide	75, 131, 73, 144, 116, 55, 128, 338, 353	STD/MS	0.074
82	15.96	3.71	79	Methyl docosapentaenoic acid	73, 55, 79, 67, 93, 117, 147, 129, 91	MS	0.029
83	16.52	3.08	371	1-Monopalmitin	73, 371, 147, 57, 129, 103, 117, 205	STD/MS	0.131
84	17.2	1.03	129	Linoleoylglycerol	73, 103, 129, 147, 117, 93, 133, 131, 337	MS	0.944
85	17.5	3.30	399	Monostearin	73, 399, 147, 57, 129, 117, 103, 203, 133	MS	0.017
86	19.22	2.23	299	Palmitoyl-lysophosphatidic acid	73, 207, 129, 55, 147, 117, 58, 77, 299	MS	0.004
87	19.49	3.23	311	Fatty acid deriv. 2	311, 73, 129, 55, 57, 67, 81, 103, 96, 93	MS	0.505
Others							
88	7.17	18.22	189	Urea	147, 189, 73, 148, 190, 171, 146, 149	STD/MS	0.021
89	7.24	2.19	179	Benzoic acid	73, 147, 58, 207, 189, 93, 171, 179, 105	STD/MS	0.034
90	7.53	43.99	299	Phosphoric acid	299, 73, 300, 314, 133, 193, 147, 211, 183	STD/MS	0.000
91	13.89	9.26	441	Uric acid	73, 456, 441, 147, 382, 458, 100, 444	STD/MS	0.085
92	16.04	1.45	202	Serotonin	73, 202, 117, 55, 147, 79, 93, 129, 290	STD/MS	0.133

(Continued on next page)

Supplemental Table 2. (Continued)

No.	Ret (min) ^z	VIP1 ^y	Unique mass (m/z)	Tentative identification ^x	MS fragment pattern (m/z)	ID ^w	P value
Unknowns							
93	9.39	2.32	327	N.I. ^u 1	73, 147, 281, 327, 415, 503, 282, 75	–	0.073
94	10.39	8.16	211	N.I. 2	73, 211, 75, 283, 129, 133, 227, 213	–	0.004
95	10.74	2.90	355	N.I. 3	73, 355, 147, 221, 75, 281, 356, 221	–	0.098
96	11.27	6.15	154	N.I. 4	73, 154, 146, 75, 156, 207, 147, 74, 91, 130	–	0.027
97	12.99	7.05	58	N.I. 5	72, 73, 58, 147, 56, 103, 133, 211, 101	–	0.037
98	15.53	2.42	221	N.I. 6	73, 147, 221, 281, 355, 75, 429, 207	–	0.079
99	16.27	1.95	355	N.I. 7	73, 147, 221, 355, 281, 207, 429, 75, 356	–	0.074
100	16.3	2.51	446	N.I. 8	73, 103, 446, 75, 147, 217, 207, 214, 447	–	0.164
101	17.87	1.45	103	N.I. 9	73, 75, 207, 147, 55, 103, 217, 129, 117	–	0.169
102	18.43	1.13	355	N.I. 10	73, 147, 221, 355, 281, 429, 295, 222	–	0.147
103	18.56	1.24	103	N.I. 11	73, 207, 147, 55, 103, 79, 129, 117, 93, 133	–	0.182

^zRetention time.^yVariable importance in the projection (VIP).^xSerum metabolite selected by VIP (>1.0) based on orthogonal partial least squares-discriminant analysis model.^wIdentification.^vComparing with standard compounds analyzed under identical analyzing condition and mass spectrum comparison with the Human Metabolome Database (2021), the National Institute of Standards and Technology database (version 2.0, 2011; FairCom, Gaithersburg, MD), and Wiley 9 database (Wiley-VCH, Weinheim, Germany).^uNonidentified.