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Airborne fungal and bacterial microbiome in classrooms of elementary schools during the COVID-19 pandemic period: Effects of school disinfection and other environmental factors

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Abstract

The aim of the study was to examine the effects of environmental factors including disinfection on airborne microbiome during the coronavirus disease 2019 pandemic, we evaluated indoor and outdoor air collected from 19 classrooms regularly disinfected. Extracted bacterial and fungal DNA samples were sequenced using the Illumina MiSeq™ platform. Using bacterial DNA copy number concentrations from qPCR analysis, multiple linear regressions including environmental factors as predictors were performed. Microbial diversity and community composition were evaluated. Classrooms disinfected with spray ≤ 1 week before sampling had lower bacterial DNA concentration (3116 DNA copies/m³) than those > 1 week (5003 copies/m³) (p -values = 0.06). The bacterial DNA copy number concentration increased with temperature and was higher in classrooms in coastal than inland cities (p -values < 0.01). Bacterial diversity in outdoor air was higher in coastal than inland cities while outdoor fungal diversity was higher in inland than coastal cities. These outdoor microbiomes affected classroom microbial diversity but bacterial community composition at the genus level in occupied classrooms were similar between coastal and inland cities. Our findings emphasize that environmental conditions including disinfection, climate, and school location are important factors in shaping classroom microbiota. Yet, further research is needed to understand the effects of modified microbiome by disinfection on occupants' health.

KEYWORDS

bioaerosol, disinfection, microbiome, NGS, PM, qPCR

1 | INTRODUCTION

Indoor air quality is important for people's health and quality of life because we spend most of our time indoors.^{1,2} Indoor air quality (IAQ) is one of the top five public health concerns of the US Environmental Protection Agency (EPA).³ Indoor air can be contaminated with chemicals and particulate matters (PMs) that include

bioaerosols. Furthermore, indoor exposure to bioaerosols has been associated with development or exacerbation of asthma and respiratory symptoms.^{4–6} Particularly, pathogenic bioaerosols including viruses can result in infectious diseases upon exposure.^{7,8}

As of late February 2022, there were more than 400 million confirmed coronavirus disease 2019 (COVID-19) cases owing to indoor infection with severe acute respiratory syndrome coronavirus 2

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(SARS-CoV-2) worldwide.⁹ To prevent spread of the infectious disease, the Ministry of Education of the Republic of Korea released guidelines for school disinfection methods in 2020.¹⁰ Disinfection methods are divided into regular and daily disinfection. Regular disinfection should be performed by a specialized company once every 2 months between April and September and once every 3 months between October and March when a long winter break is scheduled. At the end of every school day, daily disinfection is recommended to disinfect the surfaces that students frequently touch, by wiping them using hypochlorous acid (50 ppm) or 70% alcohol. However, the daily disinfection method can vary among schools by their own policies.¹⁰ Nevertheless, only limited information is available on how indoor disinfection affects the microbial communities and pathogenic microorganisms in classrooms.

Young students in elementary schools have a higher respiration rate per unit weight than adults and are likely exposed to the elevated level of bioaerosols resuspended from floors or other surfaces due to their high classroom activity.^{11,12} Recently, research in classrooms of elementary schools in Korea has been conducted to characterize indoor air quality and microbiome.^{12,13} Currently, air quality management guidelines for airborne microorganisms in Korea are based on the traditional culture method. However, it is problematic to analyze the effects of indoor environmental factors such as disinfection on microbial communities using the culture methods because 99% of the microorganisms in environments cannot be easily cultivated.^{14,15} Recently, high-throughput DNA sequencing methods became cheaper and more easily accessible than before, and thus more research on microbial communities in indoor environments using the molecular method has been conducted.^{16,17} For the current study, we selected seven elementary schools in which the classrooms were regularly disinfected during the COVID-19 pandemic in the Republic of Korea and examined the effects of classroom disinfection and other environmental conditions on airborne microbes using the DNA sequencing method.

2 | MATERIALS AND METHODS

2.1 | Selection of school regions and schools, and sample collection

Among the areas with atmospheric monitoring stations owned by the Ministry of Environment of Korea, five areas frequently exceeding the PM standard between 2015 and 2019 were selected.¹⁸ The PM_{10} ($PM < 10 \mu m$ in aerodynamic diameter) and $PM_{2.5}$ ($PM < 2.5 \mu m$ in aerodynamic diameter) standards used for the selection were the annual and 24-hour average values set by the World Health Organization (WHO, annual average $PM_{10} = 15 \mu g/m^3$, and $PM_{2.5} = 5 \mu g/m^3$; 24-hour average $PM_{10} = 45 \mu g/m^3$, and $PM_{2.5} = 15 \mu g/m^3$).¹⁹ A total of seven schools from these areas were selected—one school from each area, except for the city of Pyeongtaek. Three schools were selected in Pyeongtaek because

this area showed a higher rate of exceeding the standard than other areas during the same period (Figure S1).

Air samples were collected from a total of 19 classrooms with natural ventilation, using personal sampling pumps (Gilian Air plus, Sensidyne, St. Petersburg, USA) between June and December 2020. Three indoor sampling pumps were placed near exterior windows or on the locker located in the back of the classroom at a height of 130 cm and at least 0.5 meter away from the wall, and outdoor samples were simultaneously collected near the playground during the indoor sampling period (Figure S2). The sampling was performed during both weekdays when students were in the classrooms and weekends when students were not present (unoccupied classroom) in one of the same occupied classrooms sampled during the weekdays.¹² If sampling could not be performed in the unoccupied classroom on the weekends because access to the school was not allowed, an empty classroom next to the sampled occupied classrooms was simultaneously sampled during the weekdays. Each of the three indoor sampling pumps connected to an individual 37 mm closed cassette with a PVC filter (pore size = $5 \mu m$) collected classroom air for 7 hours from 7:30 a.m. to 2:30 p.m. during the school hour. Each of the indoor sampling pumps was operated at 2.5 L/min and outdoor sampling pump at 4.0 L/min. All pumps were calibrated before the use with Primary Pump Calibrator (Model 4146, TSI, Shoreview, USA). To ensure that sufficient genomic DNA (gDNA) for 16S rRNA gene and internal transcribed spacer (ITS) region sequencing was obtained, three indoor filters in each classroom were extracted as one composite sample, and two outdoor filters as one sample. All materials and equipment used for sampling were sterilized prior to the use with ultraviolet ray disinfection (HU-4050, Hanshin Medical CO., Incheon, Korea) for an hour to remove background DNA contamination. After sampling, the filter cassettes were sealed using parafilm and stored at $-80^\circ C$ until sample analysis. Information about schools and classrooms were collected from homeroom teachers or health teachers. This information included the number of students in the classroom, last regular school disinfection date, and daily disinfection method. Outdoor temperature and relative humidity were monitored using a digital thermo-hygrometer (TE-3203N, CAS, Guangzhou, China). Precipitation and wind velocity data were collected from the outdoor PM monitoring sites that were, on average, 500 m away (range: 200 m–1 km) from the schools.

PM_{10} and $PM_{2.5}$ in the classrooms were also monitored using a portable aerosol spectrometer (Grimm Technologies Inc., Douglasville, GA, USA) and temperature and relative humidity using a digital thermo-hygrometer (TE-3203N, CAS, Guangzhou, China). Outdoor PMs were also simultaneously monitored using a portable aerosol spectrometer (Grimm Technologies Inc., Douglasville, GA, USA) at the same locations as the bioaerosol sampling. Coarse PM (PM with aerodynamic diameter between 2.5 and $10 \mu m$, $PM_{2.5-10}$) were then calculated by subtracting $PM_{2.5}$ concentration from PM_{10} concentration. The average $PM_{2.5}$ and $PM_{2.5-10}$ concentrations for the same bioaerosol sampling time were calculated from the monitoring data for statistical analysis.

Regular disinfection was conducted by a specialist from a professional disinfection company, who used equipment to spray disinfectants (80% benzyl-C8-18-alkyldimethyl, chlorides) in the form of mists, on the weekends when there were no students in the school. The surfaces were then wiped to remove settled mists after the spraying. The elapsed time between the date of the last regular disinfection and our sampling date differed by schools and varied from 1 week to 3 months. Each homeroom teacher performed daily disinfection using 50ppm hypochlorous acid or 70% alcohol on weekdays after class when no students were present.

2.2 | Extraction of genomic DNA

The High Pure PCR Template Preference Kit (Roche, Basel, Switzerland) was used to extract gDNA from the filters. A filter was placed in a 2 ml capped microcentrifuge tube with 300mg of 212–300mm sterilized glass beads (Sigma, St. Louis, MO, USA) and 650µl of ultrapure water.²⁰ The samples were pulverized five times for 30s at 1484×g using a homogenizer (Allsheng, Hangzhou, China).^{12,21,22} After centrifugation at 20000×g for 1 min, the solution was collected and centrifuged again at 20000×g for 1 min. Next, 400µl taken from the centrifuged solution underwent lysis buffer treatment and high-temperature incubation at 70°C for 10 min. These pretreatment samples underwent a washing step using inhibitor removal buffer and wash buffer. Then, the final 100µl of gDNA was eluted and stored at –20°C until analysis.

2.3 | DNA sequencing and taxonomic identification

Sequencing was performed using the Illumina MiSeq™ platform (Illumina, San Diego, CA, USA). Each sample was prepared using the Illumina 16S rRNA amplicon sequencing library protocol. The primer sequences that were used to amplify the genes of bacterial DNA (16S V3–V4)^{23,24} and fungal DNA (ITS3–ITS4)²⁵ in the library preparation are presented in Table 1. The amount and purity of amplified DNA were measured using VICTOR Nivo (PerkinElmer, Waltham, MA, USA) and the QuantiFluor dsDNA System (Promega, Madison, WI, USA). And only samples with higher than a minimum DNA concentration (5 ng/µl) were sequenced for the 16S rRNA gene

and ITS region at a commercial laboratory (e-Biogen, Seoul, Korea) (Table S1). Pooling and regularization were performed on the results using PicoGreen (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The size of the libraries was confirmed using TapeStation DNA screen tape D1000 (Agilent, Santa Clara, CA, USA).

Sequence analysis was performed using Quantitative Insights into Microbial Ecology 2 (QIIME2, v.2020.6.), following a method suggested by Bokulich et al. (2018).²⁶ In the multiplex phase, each sequence was divided into samples using DADA2 (ver.1.11.1)^{27,28} and verified to eliminate those of low quality. The subsequent processes also used DADA2 to filter out noise sequences and correct errors in the surrounding sequence. The process of removing chimera sequences and single tones corrected the amplifier error. The clustering process used a q2-feature classifier (ver. 2020.6) tool and proceeded with an open reference operational taxonomic unit (OTU) selection method. Sequences with similarities of ≥97% were clustered into OTUs and analyzed using SILVA and UNITE.²⁹

2.4 | Quantitative analysis of bacteria

For quantitative analysis of the entire bacterial DNA, quantitative polymerase chain reaction (qPCR) analysis was performed using the primers 331F and 797R (Table 1)^{30,31} in the 16S rRNA region. In a 0.2 ml PCR tube (GmBH, Switzerland), the qPCR reaction in a total volume of 20µl was as follows: 4 µl of 5× HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia), 0.4 µl of forward and reverse primers, 1 µl of DNA template, and 14.6 µl of nuclease-free water (Qiagen, Hilden, Germany). After the initial denaturation phase (94°C, 2 min), 40cycles 15s of denaturation at 95°C, 20s of annealing at 55°C, and 30s of elongation, and data collection at 72°C were performed. LineGen 9600 (BIOER, Hangzhou, China) was used for qPCR.¹² A diluted sample of *Escherichia coli* (*E. coli*) was used as a standard curve for qPCR analysis.³⁰ PCR efficiency ($R^2 = 0.998$, PCR efficiency = 92.35%) was verified using a standard curve and its accuracy was verified using a melting curve. The concentration of bacterial DNA copy number in the sample was calculated according to the cycle threshold (Ct value) of the diluted *E. coli* sample used as the standard curve. To calculate bacterial DNA copy number in sample, we used the Ct value of diluted *E. coli* obtained from a standard curve. The OD600 value before dilution of the

TABLE 1 Primer sequences used in Illumina sequencing and quantitative polymerase chain reaction analysis

Primer			Sequence
Bacteria	V3-V4	F	5' CCTACGGGNGGCWGCAG 3'
		R	5' GACTACHVGGGTATCTAATCC 3'
	331F	F	5' TCCTACGGGAGGCAGCAGT 3'
		R	5' GGACTACCAGGGTATCTAATCCTGTT 3'
Fungi	ITS3-ITS4	F	5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGAACGCAGC 3'
		R	5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCCTCCGCTTATTGATATGC 3'

Note: Primers 331F and 797R are for qPCR and other primers are for sequencing.

Abbreviations: F, Forward primer; R, Reverse primer.

E. coli sample used to produce the standard curve was 0.7, and the corresponding DNA concentration measured after DNA extraction was 55.0 ng/ μ L. The standard *E. coli* sample was diluted 10^{-5} times. The final DNA copy number concentrations in air (copy number/ m^3) were calculated based on a linear regression line of the standard curve and the Ct value for each sample using the formula: Copy number = $10^{(C_{t_{\text{samples}}} - b)/m}$, where $C_{t_{\text{sample}}}$: Ct value for the sample; m: slope of the linear regression line; and b: intercept of the linear regression line.³²

2.5 | Diversity and statistical analysis

Microbial diversity was calculated and visualized using q2-diversity (ver. 2020.6) of the QIIME2 plugin.³³ Unidentified genera were excluded from the analysis. Rarefaction curves by school location (inland or coastal city) and sample type (occupied or unoccupied classroom, or outdoor samples) were created to examine bacterial and fungal diversity among the groups using the number of OTUs.³⁴ The similarity and composition of the microbial community in samples were analyzed by school location and sample type with the Bray–Curtis dissimilarity index, using analysis of similarity (ANOSIM) and the non-metric multidimensional scaling (NMDS), respectively.³⁵ R statistic in ANOSIM was calculated by $[4(B - W)/N(N - 1)]$, where B and W are the averages of the between-group and within-group ranks, respectively, and N is the number of samples. If schools were located in the coastal city, they were grouped into “coastal”; otherwise, schools were grouped into “inland.” For statistical modeling, schools were also categorized into “ ≤ 1 week” or “ > 1 week” depending on the elapsed time between the latest disinfection date and sampling date. Multiple linear regressions were performed to examine effects of environmental conditions on bacterial DNA copy number concentrations (copy number/ m^3). The explanatory variables included elapsed time from disinfection, sample type, temperature, relative humidity (RH), and school location. We calculated the correlation coefficients between concentrations of bacterial DNA copy number and the number of bacterial OTUs using Spearman's rank correlation method. Absolute abundance of bacteria was calculated by multiplying relative abundance with the DNA copy number determined using qPCR analysis.³⁶ All statistical analyses were performed using statistical packages vegan, tidyverse, and broom in R version 4.0 (CRAN), and statistical significance was considered at the $p \leq 0.05$ and marginal significance at the $0.05 < p < 0.1$.²⁰

3 | RESULTS AND DISCUSSION

3.1 | Classroom characteristics and outdoor weather conditions

We obtained DNA concentrations from 17 outdoor samples and 19 classroom samples (12 occupied and 7 unoccupied) (Table 2). Of these, 11 outdoor samples and 13 classroom samples (8 occupied

and 5 unoccupied) provided sufficient amount of DNA for sequencing. In five of seven schools, occupied classrooms were sampled on the weekdays and one of the same sampled occupied classrooms on the weekends (unoccupied classroom); however, in the remaining two schools, both occupied classrooms and an adjacent unoccupied classroom were simultaneously sampled on the weekdays because the schools were not open to visitors on the weekends as described earlier. The average number of students in the classroom ranged from 8 to 27 with the mean of 19.6 (standard deviation: ± 6.3). The average temperature in the classrooms ranged from 13°C to 29°C with the mean of $25.7 \pm 3.1^\circ\text{C}$. There was no difference in temperature between occupied (25.7°C) and unoccupied (24.8°C) classrooms. The average RH in occupied classrooms ranged from 29% to 60% with the mean of $50 \pm 9.5\%$. There was no difference in RH between occupied (50.3%) and unoccupied (50.7%) classrooms. The average outdoor temperature was $25.0 \pm 5.7^\circ\text{C}$, and average outdoor RH was $50 \pm 14\%$. Outdoor temperature and RH were not different from indoors. The average outdoor wind speed during the sampling days was 7.4 ± 3.39 km/h, and daily precipitation was < 7 mm for all sampling days, except for sampling at the school Ia (47.2 mm during classroom 1 sampling and 16 mm during classroom 2 sampling) (Table 2). Descriptive statistics for $\text{PM}_{2.5}$ and coarse PM are presented in Table S1. The average concentration of coarse PM in occupied classrooms was the greatest (15.5 ± 5.3 $\mu\text{g}/m^3$) followed by outdoors (10.9 ± 5.8 $\mu\text{g}/m^3$, $p = 0.08$) and unoccupied classrooms (3.6 ± 3.7 $\mu\text{g}/m^3$, $p = 0.02$) (Tables 2 and S1).

Air conditioners were operated to maintain the indoor temperature between 24°C and 26°C only when students were in the classroom. When the outdoor PM concentration was lower than the WHO standards, the room was ventilated by opening the exterior windows of the classroom as needed. When the outdoor PM concentration was higher than the standards, the interior windows toward the hallway were opened for ventilation. However, we were not able to collect information on time and the duration of natural ventilation because of restricted access to classrooms for observation during the class. Air purifiers were not used in any classrooms in accordance with the COVID-19 prevention policy.

3.2 | Effect of regular disinfection on the bacterial DNA copy number concentration in classroom air

Regular school disinfection was conducted in accordance with the Ministry of Education guidelines of the Republic of Korea in all schools. Bacterial DNA copy number concentrations of 12 occupied classrooms ranged from 90/ m^3 to 12829/ m^3 with the median of 4115/ m^3 . The elapsed time (day) between the last regular disinfection and the sampling for each school are presented in Figure 1. The elapsed time was the shortest (< 1 week) at schools Pc and Aa, while it was the longest at school Ua (3 months). Homeroom teachers in all studied schools wiped desks and doorknobs with disinfectants after classes were dismissed as a practice of daily disinfection. The teachers at school Pc additionally wiped the classroom floors

TABLE 2 Number of students for occupied classrooms and indoor and outdoor environmental factors during sampling

School	Sample type	Classroom	Number of students	Temp (°C)	RH (%)	Wind speed (km/h)	Precipitation (mm)	Average PM _{2.5} (μg/m ³)	Average PM _{2.5-10} (μg/m ³)
Pa	OC	1	25	29	50	-	-	15.13	18.64
	OUT	-	-	32	38	8.92	6.2	26.69	15.2
	OC	2	21	28	47	-	-	27.57	18.35
	OUT	-	-	31	52	10.18	0	28.97	25.22
	UOC	1	0	30	49	-	-	5.9	10.8
	OUT	-	-	30	37	17.22	1.1	3.95	10.76
Pb	OC	1	17	26	55	-	-	25.74	9.56
	OUT	-	-	29	49	5.16	0	26.44	15.24
	OC	2	18	24	49	-	-	36.6	8.86
	OUT	-	-	25	61	5.28	0	33.09	14.18
	UOC	1	0	28	55	-	-	25.17	3.07
	OUT	-	-	32	40	3.88	0	15.11	3.5
Pc	OC	1	23	28	51	-	-	35.01	10.9
	OUT	-	-	30	45	5.91	0	17.13	6.45
	OC	2	25	28	50	-	-	40.82	15.15
	OUT	-	-	31	45	4.06	0	21.4	7.11
	UOC	1	0	28	57	-	-	28.96	2.64
	OUT	-	-	28	56	7.29	0	18.13	6.43
Aa	OC	1	8	26	60	-	-	.*	.*
	OUT	-	-	27	62	5.58	0	.*	.*
	OC	2	8	27	60	-	-	.*	.*
	OUT	-	-	32	46	5.84	0	.*	.*
	UOC	1	0	28	61	-	-	.*	.*
	OUT	-	-	30	53	6.08	0	.*	.*
Ia	OC	1	27	27	52	-	-	3.69	14.79
	OUT	-	-	21	86	11.16	47.2	5.86	10.81
	OC	2	20	25	62	-	-	4.16	25.29
	OUT	-	-	22	72	7.76	16.2	6.76	9.91
	UOC	1	0	27	62	-	-	19.26	1.38
	OUT	-	-	28	56	10.12	0	2.62	2.72
Da	OC	1	25	22	37.5	-	-	8.29	12.31
	UOC	2	0	22	41.5	-	-	5.5	0.5
	OUT	-	-	22	28.5	4.44	1.2	8.37	13.99
Ua	OC	1	18	18	25.5	-	-	22.36	20.97
	UOC	2	0	13	38.5	-	-	14.41	3.4
	OUT	-	-	14	35.5	7.32	0.7	23.29	11.01

Abbreviations: OC, occupied classroom sample; OUT, outdoor sample; PM, particulate matter; RH, relative humidity; Temp, temperature; UOC, unoccupied classroom sample.

Note: Schools Da and Ua were not accessible during the weekends. Therefore, occupied classrooms and an adjacent unoccupied classroom were sampled on the same day during weekdays. Schools Pa, Pb, Pc, and Ua are from coastal cities.

* indicates instrument failed during monitoring PM concentrations.

using a wet mop after class during weekdays. We were not able to obtain enough amount of DNA for the sequencing analysis in the samples from schools Pc and Aa. The shortest elapsed time (<1 week) from the regular disinfection with subsequent removal of

surface dust by wiping for these two schools might have decreased bioaerosols and resuspension of dust from floor and elevated surfaces in these schools, resulting in low amplified DNA concentrations (<5 ng/μl).

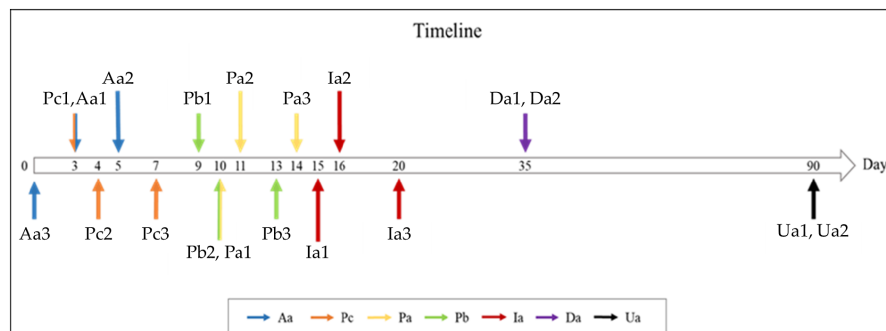


FIGURE 1 Elapsed time between the date of the last regular disinfection and the sampling date. School IDs: Pa, Pb, Pc, Ia, Da, and Ua, and the following numbers denote classroom ID

In crude analysis of the bacterial DNA copy number concentration in the occupied classroom air by school location, concentrations ($1678 \pm 483/\text{m}^3$) in classrooms in inland cities that received the last regular disinfection less than a week before the sampling was lower than those ($2605 \pm 394/\text{m}^3$) that were disinfected more than a week before sampling, but the difference was not statistically significant ($p = 0.23$). In the classrooms in coastal cities, the concentration received the regular disinfection ≤ 1 week before sampling was slightly higher ($9926 \pm 2752/\text{m}^3$) than that >1 week ($8485 \pm 1740/\text{m}^3$), and the difference was not significant ($p = 0.67$). However, in multiple linear regression models using all the measurements ($N = 32$) adjusted for school location, sample type, temperature, and RH, bacterial DNA copy number concentrations in classrooms disinfected ≤ 1 week before the sampling were lower than those disinfected >1 week ($3116 \pm 712/\text{m}^3$ vs. $5003 \pm 590/\text{m}^3$) and the difference was marginally significant ($p = 0.056$) (Table 3). With the increased sample size, the statistical significance might have been improved. Our findings imply that the regular disinfection with the spraying method may have decreased airborne bacterial microbiota. This is likely

because the mists sprayed with disinfectants might have cleared the airborne bacteria from air through settlement with the sprayed mists on indoor surfaces and then subsequently removed from the classrooms by wiping the surfaces as discussed earlier. Previous publications in the literature reported that airborne bacterial and fungal concentrations determined using a culture method in disinfected fitness center or hospital operating rooms were significantly lower than those before disinfection.^{37,38} Unfortunately, we were not able to examine diversity or microbial community composition among the classrooms grouped by elapsed time from the regular disinfection. This was because we did not have sequencing results because of the low amount of DNA for the schools with shorter elapsed times of disinfection (≤ 1 week). However, we found that bacterial DNA copy number concentrations were significantly and positively correlated with the number of bacterial OTUs (Spearman $r = 0.48$, $p = 0.02$), which might also indicate low bacterial diversity in the classrooms disinfected within 1 week before the sampling compared with those disinfected >1 week.

TABLE 3 Effects of environmental factors on concentrations of bacterial DNA copy number

Environmental factor (continuous or categorical) ^a	Bacteria DNA copy number/ m^3 (p -value)
Disinfection time	
≤ 1 week (Ref)	3116
>1 weeks	5003 (0.06)
Sample type	
Unoccupied (Ref)	2913
Occupied	5506 (0.02)
Outdoor	3759 (0.61)
Temperature ($^{\circ}\text{C}$)	297 (<0.01)
Relative humidity (%)	-28 (0.44)
Location	
Inland city	1827
Coastal city	6292 (<0.001)
Adjusted Model R^2	0.603

^aThe effects are model coefficients for continuous variables and least squares means for categorical variables. Bolded numbers: Statistically significant effect. Italicized numbers: Marginally significant effect. Ref: Reference group in categorical variables.

3.3 | Effects of environmental factors on the concentrations of airborne bacterial DNA copy number

Multiple linear regression model indicated that the mean concentration of bacterial DNA copy number in the occupied classroom air was significantly higher than that in the unoccupied classrooms (Table 3). Air temperature had significant and strong positive effect on the concentrations of bacterial DNA copy number; however, air RH did not have a significant effect on the concentrations. This may indicate that airborne bacterial microbiota is more responsive to temperature increase than humidity. Our findings seem to be supported by other published studies.^{39–41} In a study of microbial communities in the tropical air ecosystem, air temperature was positively correlated with bacterial abundance in all phyla.³⁹ Ma et al.'s study of bacterial communities in marine aerosols also found that temperature was a significant factor affecting bacterial community structure.⁴¹ From the multiple regression model, we also found that the adjusted mean air concentration of bacterial DNA copy number in the classrooms in coastal cities was 3.4-fold significantly ($p < 0.001$) higher than that in inland cities. However, microbial diversity and community compositions in occupied classrooms by school location were complex and

discussed in the next section in detail. To increase model efficiency with the maximum sample size, we excluded PM measurements from the regression model that had missingness in six samples. However, the crude regression analyses examining associations between the concentrations of bacterial DNA copy number and PM concentrations by school location indicated that the DNA copy number concentrations were significantly and positively associated with the coarse PM (coefficient = 328 DNA copies/m³ increase per 1 µg/m³ coarse PM, $p = 0.02$) and marginally with PM_{2.5} concentrations (210 DNA copies/m³ increase per 1 µg/m³ coarse PM_{2.5}, $p = 0.08$) only in classrooms in coastal cities but not positively associated with any PM concentrations in those in inland cities. This result may indicate that coarse PM concentration may be more closely associated with bacterial concentration than fine PM (PM_{2.5}); however, such association may depend on school location such as coastal or inland cities. Unfortunately, we could not perform fungal qPCR analysis due to an issue in the logistics of the experiment.

3.4 | Bacterial and fungal diversity and community composition by school location and sample type

Rarefaction curves of bacterial OTUs by sample type (occupied, unoccupied, and outdoor) are presented in Figure S3 and by both sample type and school location (coastal versus inland) in Figure 2. The rarefaction curves demonstrated sufficient sequencing depth by reaching the zero-slope asymptotic line, except for outdoor air in coastal cities showing slow and continuous increase in the number of bacterial OTUs. The number of bacterial OTUs from the rarefaction curves identified in the compiled all outdoor sample data was the greatest (2829) followed by all occupied classroom (2080) and compiled unoccupied classroom (692) (Figure S3A). However, rarefaction curves grouped by both location and sample type (Figure 2A) indicated that for coastal cities the number of bacterial OTUs in outdoor samples was much higher than that of occupied classrooms while for inland cities the number of OTUs in outdoor samples was not much different from occupied classrooms. The number of bacterial OTUs in occupied classrooms was higher in coastal than inland city schools, implicating the influence of high outdoor OTUs of coastal city on indoor bacterial diversity. However, the NMDS plot at the genus level showed that the bacterial community composition of occupied classrooms in coastal cities was similar to inland cities while outdoor genus composition in coastal cities was different from inland cities (Figure 2B). The ANOSIM analysis at the genus level also showed similarity in bacterial community composition in occupied classrooms between two locations (R statistics = -0.04 , $p = 0.53$), but dissimilarity in outdoor air between coastal and inland cities (R statistics = 0.50 , $p = 0.01$) with higher variability in outdoor air of coastal cities (Figure 3a,b). These findings may indicate that high levels of outdoor bacterial OTUs affected indoor microbiome of the studied classrooms with natural ventilation at the species or sub-species level. However, similarity in bacterial composition shown in the NMDS plot at the genus level indicates that occupants may still

remain an important source of classroom bacteria.^{12,42} All schools in coastal cities were within 12 km from the ocean while the schools in inland cities were at least 29 km away from the ocean. Thus, the schools in coastal cities might have been affected by bacteria-rich marine aerosols that were transported from sea surface to air.⁴³

The rarefaction curves of fungal OTUs showed similar results as bacteria. The number of OTUs identified in the compiled all outdoor sample data was the highest (3091) followed by compiled occupied classrooms (2348) and unoccupied classrooms (936) (Figure S3B). However, in contrast to bacterial diversity, the number of fungal OTUs in outdoor air was higher in inland cities than coastal cities (Figure 2C). In both coastal and inland cities, the number of fungal OTUs in occupied classrooms was lower than outdoors but the difference between occupied classrooms and outdoors was much greater in inland cities than coastal cities. At the genus level, fungal community compositions in occupied classroom or outdoor air were different between inland and coastal cities (Figure 2D). The ANOSIM results for fungal DNA indicated insignificant difference in dissimilarity (R statistics = 0.17 , $p = 0.24$) in fungal genus composition of occupied classrooms between two locations but substantial dissimilarity ($R = 0.50$, $p < 0.01$) in composition of outdoor air between two locations. High outdoor fungal diversity in inland cities may be influenced by the results of the inland school 'la', which received more rain during sampling (Table 2) conducted during the summer monsoon in Korea. Rain during the monsoon in Korea substantially increased outdoor bioaerosol concentrations with a stronger effect on fungi than bacteria.⁴⁰ Conversely, Salonen et al. observed the highest fungal concentrations in classrooms when humidity was low but temperature was high from a study of elementary schools in Brisbane, Australia.⁴⁴ NMDS plots at the genus level also indicated that fungal community composition in occupied classrooms is closely related to their outdoor air composition in both locations as we found in a previous study.¹² Salonen et al.'s meta-analysis to estimate mean indoor and outdoor culturable fungal concentrations by different seasons and climate regions found that the climatic condition most importantly affected airborne fungal concentrations in school environments.⁴⁵

The stacked bar graphs in Figure 4 show relative and absolute abundance of the 20 most abundant bacterial genera and relative abundance of the top 20 fungal genera. For bacteria, *Staphylococcus*, *Corynebacterium*, and *Sphingomonas* were the top three most abundant genera in occupied classrooms of schools in coastal cities while *Staphylococcus*, *Micrococcus*, and *Paracoccus* were the top three in inland cities. *Staphylococcus*, *Corynebacterium*, *Paracoccus*, and *Micrococcus* that were observed in the occupied classrooms in our study have also been observed in the community of human skin microorganisms in multiple other studies.^{7,46,47} *Staphylococcus* was the most abundant in occupied classrooms in both cities that is the dominant bacterium on hands and legs in human body, and *Corynebacterium* on elbow wrinkles.⁴⁶ For outdoor airborne bacteria, *Bacteroides*, *Lactobacillus*, and *Methylobacterium* were the most abundant in coastal cities while *Staphylococcus*, *Cutibacterium*, and *Enhydrobacter* were the most abundant in inland cities (Figure 4A).

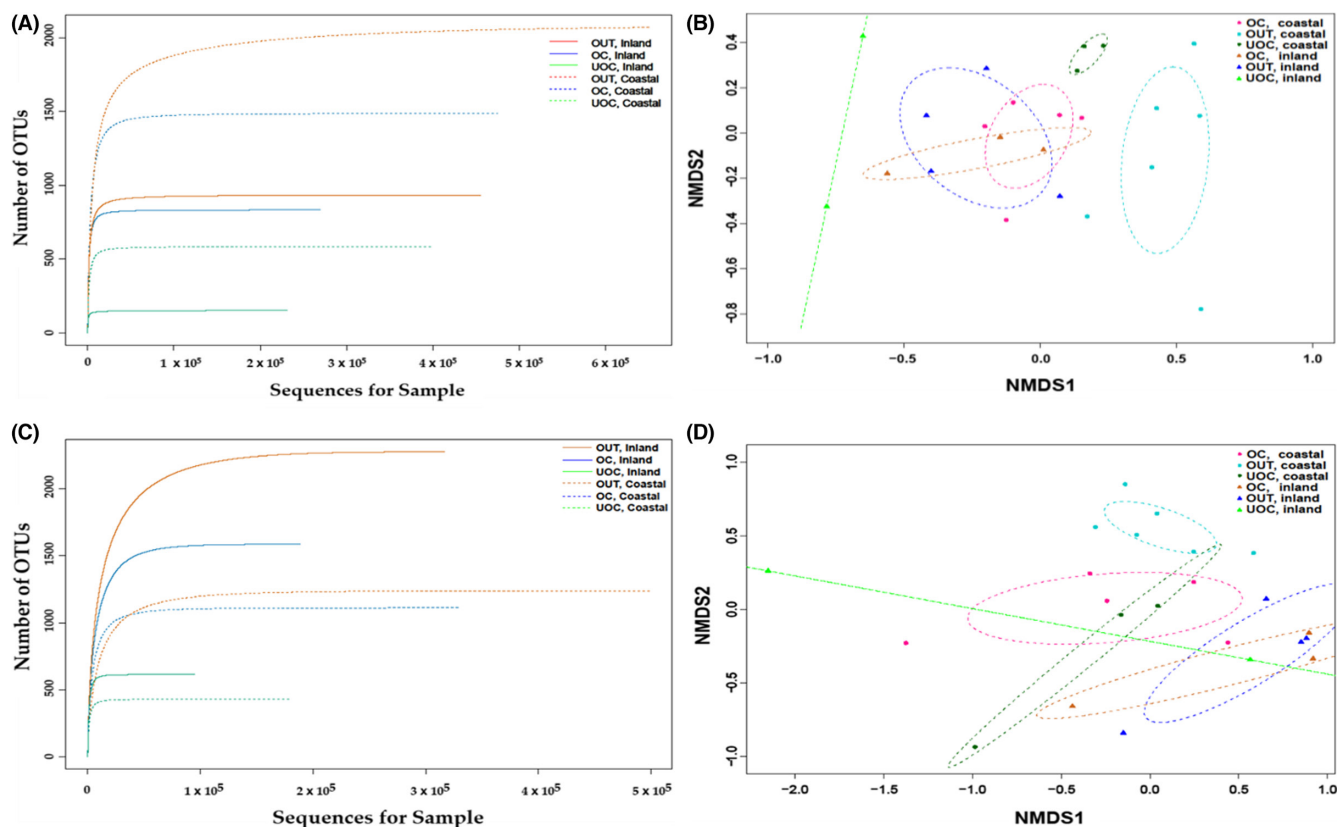


FIGURE 2 Rarefaction curves (A) and non-metric multidimensional scaling (NMDS) using the Bray–Curtis dissimilarity index (B) at the genus level for bacteria by school location and sample type; rarefaction curves (C) and non-metric multidimensional scaling (NMDS) using the Bray–Curtis dissimilarity index (D) at the genus level for fungi by school location and sample type. OC, occupied classroom; UOC, unoccupied classroom; OUT, outdoor, dotted ellipse: 95% confidence interval

Bacteroides and *Methylobacterium* have been reported to be abundant in outdoor air of the coastal cities.^{41,48} The top 10 most abundant bacterial genera from the analysis using relative abundance remain as the top 10 from the analysis using absolute abundance, except for *Burkholderia* (Figure 4B). DNA copy number concentrations of these top 10 most abundant bacteria were lower than 2400 copy number/m³ of air. For fungi, *Cladosporium* was the dominant genus both indoors and outdoors (Figures S3C and S4). *Cladosporium* and *Alternaria* were the most abundant genera in occupied classrooms and outdoor air in coastal cities; however, *Cladosporium*, *Peniophora*, and *Irpex* were the most abundant genera in occupied classrooms and outdoors in inland cities. *Alternaria* and *Cladosporium* that were dominantly found in occupied classrooms of schools in coastal cities are among the fungi associated with allergic diseases such as rhinitis and asthma.^{49,50}

One of the limitations of our study is a small sample size. Because of restricted school access during the pandemic, it was extremely challenging to increase the number of schools and classrooms. The small sample size did not provide enough statistical power for more comprehensive statistical modeling. Also, the regular disinfection within a week before sampling significantly affected amount of amplified DNA for further DNA sequencing, which resulted in the decreased number of samples available for the analysis of community composition. Nonetheless, using the concentrations of bacterial

DNA copy number obtained from the qPCR analysis, we were able to observe the effects of disinfection and other environmental factors on indoor microbes. A limitation in our qPCR-based quantitative taxonomic profiling would be potential biases from the use of different primers for qPCR analysis and the DNA sequencing. However, primer coverage between the qPCR and sequencing primers was also reported to be comparable.³⁶ The qPCR-based quantitative taxonomic profiling in the future study could be further improved by using the same primers in both methods. Our study used different flow rates by sample types (i.e., 2.5 L/min for each of the three pumps for indoor samples and 4.0 L/min for each of the two pumps for outdoor samples) to ensure that outdoor air sampling collects similar total volume of air to indoor air sampling with fewer pumps. A study reported that using different sampling flow rates (100, 200, or 300 L/min) caused different particle retention efficiency;⁵¹ however, our study used about 2 orders of magnitude lower flow rates, which would not likely to cause a significant difference in collection efficiency between the indoor and outdoor samples, as found in a study using low flow rates (1 to 4 L/min).⁵² Lastly, the controlled laboratory studies to further examine the effect of disinfection on indoor microbial community composition and how long the effect would last may improve our understanding of effectiveness of disinfection in school environments and its implication for occupants' health.

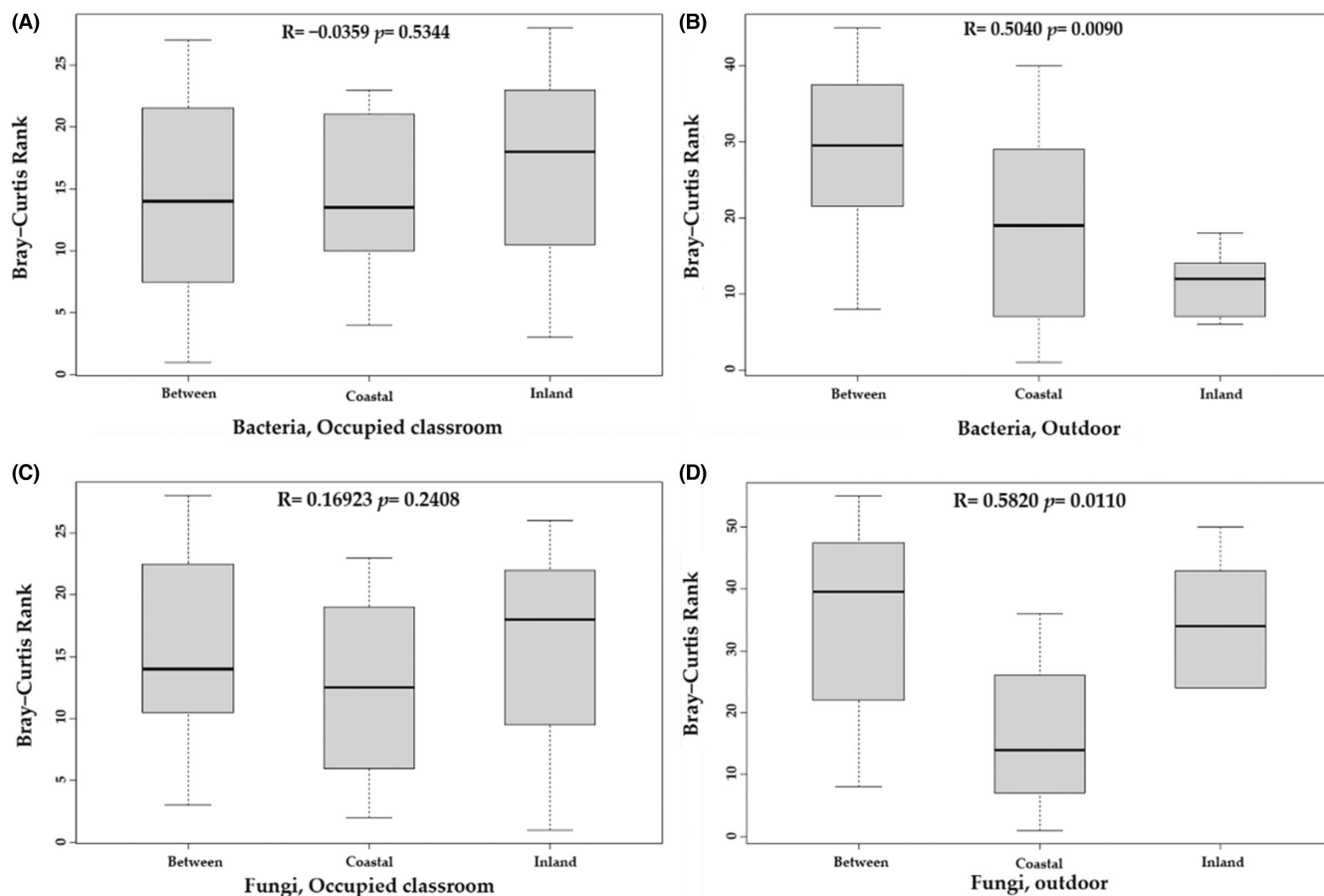


FIGURE 3 Analysis of similarities (ANOSIM) at the genus level of bacteria for samples in occupied classrooms (A) and outdoor samples (B) by school location; at the genus level of fungi for samples in occupied classrooms (C) and outdoor samples (D) by school location

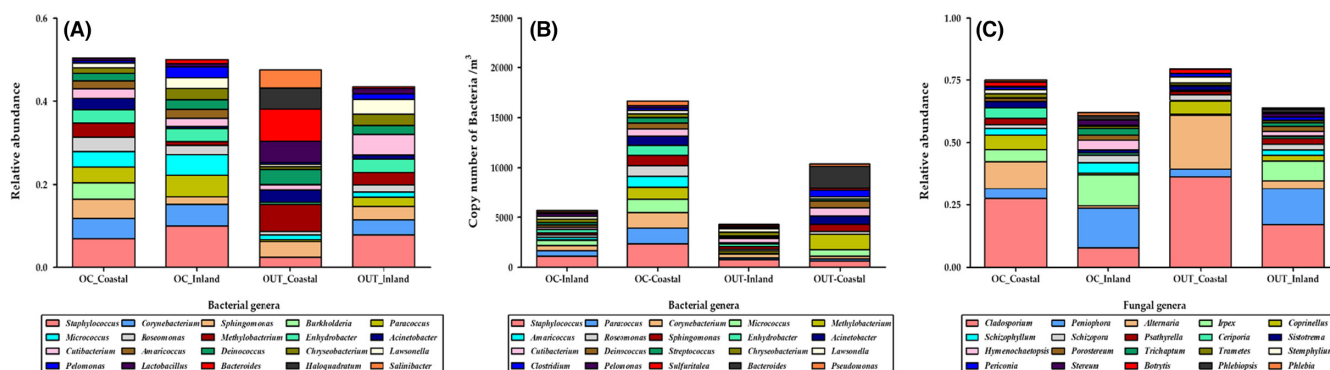


FIGURE 4 Relative abundance of bacteria (A), absolute abundance of bacteria (B), and Relative abundance of fungi (C) present in school classrooms and outdoors. OC, occupied classroom; OUT, outdoor sample

4 | CONCLUSIONS

From our school study during the COVID-19 pandemic, we observed that regular disinfection with spraying disinfectant along with subsequent wiping of indoor surfaces in school classrooms may decrease airborne bacteria. We also observed that airborne concentrations of coarse PM, especially in coastal cities, were more strongly and positively associated with airborne concentrations of bacterial DNA

copy number than fine PM. In addition, air temperature was significantly and positively associated with the concentration of bacterial DNA copy number in air. Indoor coarse PM concentration and temperature in classrooms may be considered important environmental factors that can control indoor air microbiota, especially bacteria. Classroom microbiome was also largely affected by school location such as coastal or inland cities, indicating that where the schools are built is also likely an important determinant of school microbiota.

Epidemiologic studies of schools from both coastal and inland cities in dry and rainy seasons will enlighten the effects of different microbiota by school location and season on occupants' health. In addition, more research is needed to better understand implications of modified classroom microbiota by school disinfection on occupants' health.

AUTHOR CONTRIBUTIONS

For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used: Conceptualization, validation, and writing—review and editing: J.P. and M.Y.; methodology: J.P., B.L. and J.Y.; formal analysis: J.P. and J.Y.; investigation: J.Y. and B.L.; data curation, writing—original draft preparation, visualization: J.Y.; supervision, project administration, funding acquisition: M.Y. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DISCLAIMER

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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