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Investigation of Surface Bacterial Diversities and Compositions in the Global Subway Facilities

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Abstract: Indoor microbes are a key component of air contamination that causes human health risks. However, compared with the aquatic and soil environment, microbial diversity and taxonomic structure and composition in subway facility are not well characterized. This study tries to explore surface bacterial communities by using swabs collected from four global subway facilities, such as Busan, Boston, Mexico City, and Moscow using 16S rRNA gene amplicon sequencing. The alpha-diversities on bacterial communities were significantly different between Moscow and other samples, despite the different sample characteristics among Busan, Boston, Mexico City samples. For bacterial taxonomic composition, three phyla such as Actinobacteria (41.1%), Proteobacteria (27.7%), and Firmicutes (18.9%), were most dominant among all samples, indicating that there was no significance ($p > 0.05$). The subway station surface samples were mostly dominated by Gram-positive bacteria, including genera *Corynebacterium*, *Staphylococcus*, and *Streptococcus*. PCoA analysis also revealed that the Moscow bacterial communities were clearly separated from others. In addition, core genera were only shared 75 genera among all samples, but 486 genera were shared with three global stations, such as Busan, Boston and Mexico City. These results suggested that the human activity and geographical environment potentially affect the establishment of the bacterial community. Although this study provided basic information on surface bacterial communities in the subway system, there is a remaining unknown microbiome in the indoor air environment. Therefore, we consistently try to understand the indoor environment's microbial ecology in the subway system.

Keywords: surface microbial community; subway microbes; indoor microbiome; high-throughput sequencing

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1. Introduction

Despite the adverse impact of suspended microorganism exposure on human health [1–4], relatively little is known regarding microbial particles in indoor facilities. In general, indoor transportation facilities used by an unspecified number of travelers can lead to exposure to different pollutants from those in homes or offices that are typically occupied by the same people for the same time periods [5,6]. The assessment of indoor air quality and exposure of commuters to air pollution in transportation facilities has garnered increasing attention due to its public health implications [7–9]. However, most studies on airborne substances have investigated the chemicals present in indoor air and have mostly focused on physiochemical hazards and risks, whereas a comprehensive exploration of the microbial community in multiple-use indoor places has not been conducted [6,10,11].

Currently, our knowledge of indoor microbial communities related to urban environments is less than that of natural ecosystems. As part of the city's infrastructure, subway plays a very important role in the daily lives of countless people, but it can be one of risk indoor space that is very sensitive to the spread of bioaerosol and the spread of infectious

diseases [6,12,13]. Biological particles floating in the air of the subway may be derived from microbiomes on the surface of indoor facilities, and microorganisms may be deposited on various indoor surfaces to provide microbiome information in the indoor space [5,14]. Since deposited dust can be resuspended to form airborne microbial particles following ventilation systems and human movement, surface microbial community survey is suggested as one method of determining indoor air quality [6,15]. Metro users constantly interact with each other and suspended unique indoor microorganisms. At that time, humans can become a major source such as native microbes and carriers of foreign microbes, acting as potential targets for opportunistic species [2,16]. Previous studies related to Metro indoor air microbial communication also reported that human-derived microorganisms dominate the surface of indoor facilities of subway [17–20]. In addition, previous studies highlighted that the metro microbiome potentially provided specific geographical characteristics, such as daily temperature differences, differences between communities occupying gaps of various substances in subway locations, as well as potential pathogen emergence and microbial community composition [5,16,19].

The investigation of microbial communities associated with urban subway systems are of particular interest for microbial ecology and environmental health aspect, as well as air environmental research [5,6]. Additionally, specific information on microbial communities of urban subway systems can be contributed to the understanding of ecological indicators and their niche in urban subway system. However, such information on the colonization of the subway microbiome is still lacking even though many previous pollution studies have been conducted on global metropolitan areas. Therefore, the aim of this study was to advance the knowledge of urban metro microbiome through analysis of global subway system. We compressively analyze the spatial bacterial community according to the geographically, culturally, and economically different metropolitan cities to investigate alpha, beta-diversities and major bacterial taxa.

2. Materials and Methods

2.1. Study Sites and Sampling

Busan is one of the metropolitan cities in South Korea, and the Busan subway and airport are daily crowded with commuters and tourists. Surface samples of public transportation facilities were collected from five subway stations (BS1–BS5) between 25 June and 11 August 2021. The detail information on subway stations was previously reported [20]. Briefly, subway stations were transited by approximately 15,000~29,000 passengers daily [21], and the airport, a major transportation hub with a wide range of traffic, has a daily population of approximately 400,000 [22]. The Boston Subway operates approximately 238 million trips a year and is one of the four largest subway systems in the USA, transporting more than 1 million passengers per weekday [19,23]. The subway system of Boston city is transited by approximately 520,000 passengers each day [24]. Mexico City, the largest city in the Western, has a population of 21.3 million [18], and its subway is the ninth busiest system in the world [25]. The subway system of Mexico City in Mexico has a user population of more than 4 million every day [26]. The population of Moscow is 17 million people, and the Moscow subway is the sixth most used in the world [16,27]. The subway system of Moscow city in Russia has a daily user population of approximately 7 million [28]. Each city of average summer temperature is approximately 23.9 °C in Busan, 21.7 °C in Boston, 17.7 °C in Mexico City, and 17.2 °C in Moscow [29]. Boston is characterized by a climate with four distinct seasons and an even distribution of precipitation throughout the year, with an average summer precipitation of about 88.6 mm [30]. Busan, Mexico City, and Moscow are characterized by most of the annual average precipitation occurring in summer, with average summer precipitation of approximately 239.8 mm, 159.7 mm, and 82 mm, respectively [31,32].

In this case, 15 sequences of subway station surface each were downloaded from previous studies in Boston (BT1–BT5), Mexico City (MX1–MX5), and Moscow (MC1–MC5), as shown in Table 1. Sequences were selected when they satisfied all three conditions as

follows: (1) indoor subway station sample, (2) surface swab sample, and (3) 16S rRNA gene sequences for targeting the V4 region. In addition, this study considered the number of subway users, urban size, climate, and geographical location (including latitude and longitude) when selecting the sample for this study. Since the Busan sample was collected only from subway stations, not subways, sequences from other studies were also limited to those from subway stations. The selected sequences were filtered again to make the surface material and location similar to that of the Busan, and the same number of samples were selected at Boston, Mexico City, and Moscow. Since all three cities have distinct four seasons and an even distribution of annual precipitation, all sequences used in the study were determined at similar sampling times from summer to early fall. Microorganisms in the air in the indoor environment have limited circulation of outside air compared to outdoors. They have relatively low exposure to ultraviolet rays of sunlight, enabling long-term survival [1,2]. In addition, indoor environmental factors such as temperature, humidity, and carbon dioxide (CO_2) are reported to significantly correlate with microbial diversity and composition formation [4–6]. This study attempted to investigate the relationship between these indoor environmental factors with global subway stations. However, the available data were limited, and some studies did not measure indoor environmental factors, making it difficult to perform additional statistical analysis.

Table 1. Samples and obtained sequences information in this study.

City	Site	Station	Sequence Available
Busan	BS1	Busan Station	mgs860663
	BS2	Seomyeon	mgs860666
	BS3	Busan National University	mgs884070
	BS4	Haeundae	mgs884082
	BS5	Airport	mgs860669
Boston	BT1	Alewife	SRR3498906
	BT2	Riverside	SRR3545943
	BT3	South(underground)	SRR3545897
	BT4	South(upstairs)	SRR3545957
	BT5	Foresthills	SRR3545889
Mexico City	MX1	Indios verdes	SRR9671870
	MX2	Pantitlan	SRR9671879
	MX3	Tacubaya	SRR9671874
	MX4	Buenavista	SRR9671878
	MX5	Tacuba	SRR9671883
Moscow	MC1	Rimskaya	SRR7976670
	MC2	Sretenskiy boulevard	SRR7976673
	MC3	Vystavochnaya	SRR7976678
	MC4	Vystavochnaya	SRR7976679
	MC5	Dostoyevskaya	SRR7976692

Indoor surface samples for Busan city were collected two times per site using Isohelix DNA/RNA buccal swabs (SK-2S, Isohelix) moistened with sterile 1 mL of phosphate-buffered saline. Samples were collected for 3 min from each site in a 100 cm^2 area as recommend in previous study [33] of the surface structures, such as door gate, drawers, and storage boxes. After swab sampling, the swab head into new empty 1.5 mL microtubes and transported to the laboratory in an icebox. The samples were stored at −20 or −80 °C until further analysis.

2.2. DNA Extraction and 16S rRNA Gene Sequencing

The collected two swab samples cut into 2 mm pieces were placed in a bead tube of an extraction kit and added lysis buffer. The tubes were incubated at 65 °C for 10 min using heat block (IKA Dry Block Heater 2 with DB 1.2) [34]. DNA extraction was conducted using the DNeasy PowerSoil Pro Kit (QIAGEN) with a slightly modified Quick-Start

Protocol (QIAGEN). The wash process was performed one more time to enhance DNA purity. Afterward, DNA yield and quality were measured using a Nano-300 UV-vis microspectrometer (Allsheng, China). Prior to further experiment and 16S rRNA gene sequencing, the extracted DNA was stored at −80 °C.

The suspended indoor microbiome was detected based on the 16S rRNA gene. The V3–V4 region of the 16S rRNA gene was amplified using the 341F (5′-CCT ACG GGN GGC WGC AG-3′)/805R (5′-GAC TAC HVG GGT ATC TAA TCC-3′) primer set. Amplicon libraries were constructed following the Illumina MiSeq platform as 2 × 250 bp paired-end protocol. Sequencing was carried out Macrogen (Seoul, Korea), and amplicon sequences are deposited in the MG-RAST under sample IDs mgs860663, mgs860666, mgs884070, mgs884082, mgs860669.

2.3. Bacterial Communities Analysis

After checking the quality of amplicon sequences using FastQC [35], Trimmomatic (version 0.33) was used to trim ambiguous or low-quality adaptor sequences (<Q30) and to remove potential duplicate reads due to amplification artifacts [36]. The Mothur software (version 1.44.3) was used to sequence alignment, classification, and OTU (operational taxonomic unit) clustering following the MiSeq SOP [37,38]. OTUs were defined sequences with >97% similarity, and representative sequence for each OTU was determined using Mothur algorithm for taxonomic annotation. The SILVA reference database was used to assign taxonomic profiles [38]. The alpha and beta diversity of bacterial communities was analyzed using Mothur algorithms [37,38].

2.4. Statistical Analyses

The 'phyloseq' package in R [39] was used to analyze bacterial communities. Principal coordinate analysis (PCoA) based Bray-Curtis dissimilarity was visualized with the 'ggplot2' package [40]. The upset plot was visualized with genus level, using the 'UpSetR' package [41]. All statistical significance was analyzed with the 'vegan' package in R [42]. The significance among microbial communities in four countries was assessed via Wilcoxon-signed rank tests. In order to identify the statistically significant influence of sampling site on bacterial community structures, one-way analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) test was performed with considered statistically significant (p-value < 0.05).

3. Results and Discussion

3.1. Surface Bacterial Diversity from Different Subway Stations

Bacterial diversities were calculated alpha diversity using genera relative abundance and, estimated using the ACE, Chao1, Shannon, and Simpson indices (Figure 1). The highest diversity was found on BT samples, while the lowest diversity station was found on MC samples. The bacterial diversities of both the BS, BT, and MX samples were significantly higher than the MC diversities [mean Shannon index values of 4.59 (BS), 5.01 (BT), 4.07 (MX), and 2.13 (MC), respectively; $p < 0.05$]. The most diverse station was BT, with a mean ACE diversity index of 1131.61, followed by BS (872.96), MX City (632.92), and MC (110.03). The highest Chao1 diversity was found on BT (1131.57), followed by BS (879.26), MX (634.267), and MC (107.27). The highest Simpson diversity was found on the BT (0.98), followed by BS (0.97), MX (0.95), and MC (0.81). The alpha diversity indices had similar distribution patterns, with the MC samples showing low values than the other samples, there had a significant difference ($p < 0.05$). Although the diversity of BS samples showed higher than the diversity of MX and lower than the diversity of BT, there was no significant difference ($p > 0.05$).

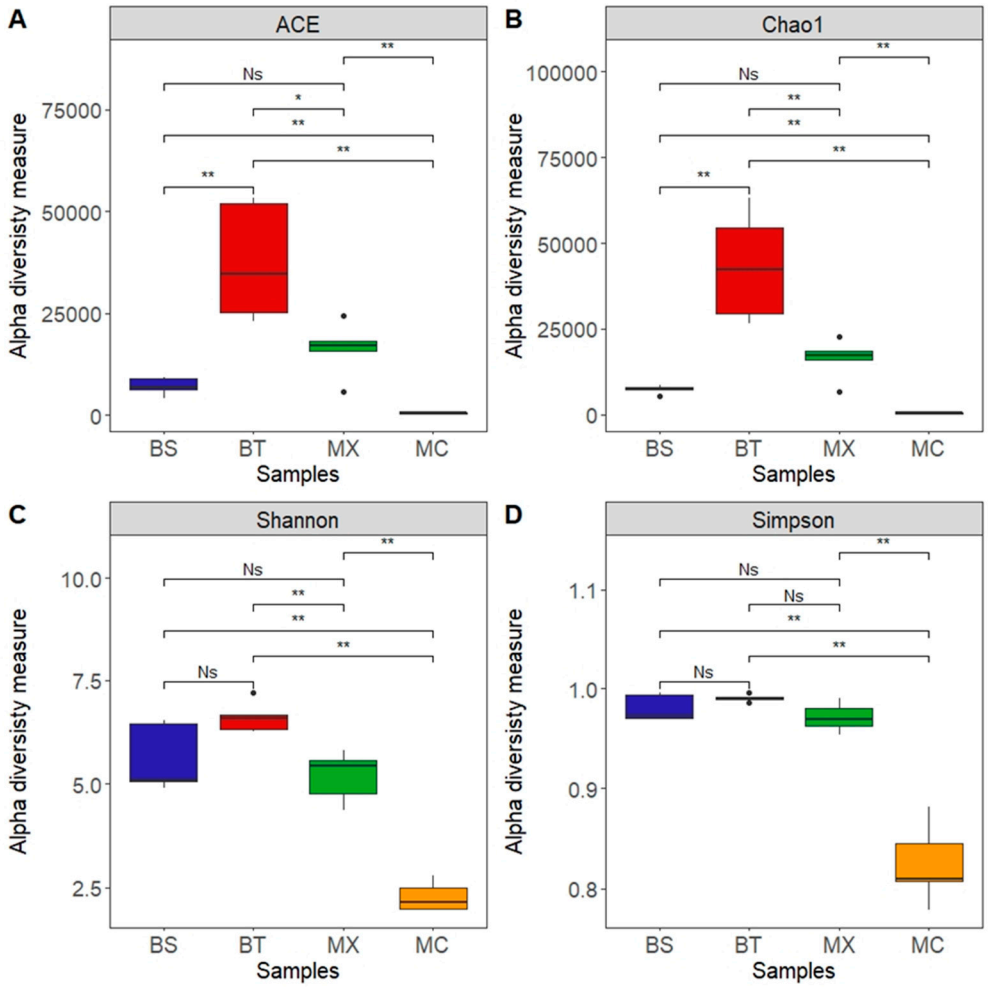

Figure 1. Alpha diversity was shown in each city. Blue: Busan, Red: Boston, Green: Mexico City, Orange: Moscow. Statistically significant differences between the two sample types were shown as follows. **: $p < 0.01$, *: $p < 0.05$, NS: Non-Significant.

The bacterial diversity of BS has similarities to the BT and MX, and significant differences in bacterial diversity from MC. These results suggested that microbial diversities were potentially affected by geographic climate. Previous studies consistently indicated that the geographical distribution of facilities might be an important and influential variable in the variance of indoor microbial communities [43,44]. The bacterial diversity and evenness may reflect not only the geographic climate but also the cultural, social, and environmental differences of each region [4,18,45]. In addition, commuters could positively contribute the increasing of bacterial diversity [46,47]. Although the mechanisms causing this phenomenon have not been defined, the surface bacterial diversity in indoor spaces may be influenced by complex factors such as overpopulation, air environmental parameters, sampling time, and ventilation systems [48–51].

To identify differences between our study and previous studies, we compared the alpha diversity with subway bioaerosol studies. Previous study has shown that the Chao1 value of the Hong Kong subway was approximately 2200 [52], which is higher than MC samples but lower than other samples. The Shannon index values for the Barcelona metro range from 1 to 2 [53], which is lower than the lowest MC sample in our study. On the other hand, the Oslo subway showed a Shannon index of about 6.3, which were similar to that of BT [5]. As indicated, bacterial diversities were quite diverse, according to the urban cities. These results suggested that because the indoor air quality parameters, such as temperature and humidity levels were not consistently measured, and very complex indoor environmental mechanisms and relationships with microbes were still black-box, it is

difficult to interpret these inconsistent observations [4,54]. The main air quality parameters generated inside subway stations are PM, CO_2, VOCs, and bioaerosols. Ozone (O_3) and carbon monoxide (CO) originate outside subway stations and infiltrate these areas [55]. The environmental parameters of indoor air quality are highly linked between changes in microbial diversity with surrounding air conditions [56]. Therefore, these air parameters should be determined to better understand potential relationships between microbial communities and environmental characteristics in indoor subway facilities.

3.2. Surface Bacterial Community Composition from Different Subway Stations

The bacterial taxonomic compositions from global subway samples were showed in Figure 2. The most abundant phyla in all samples were Actinobacteria (mean 41.1%), Proteobacteria (mean 27.7%), and Firmicutes (mean 18.9%), accounting for approximately 88% of the total phyla (Figure 2A). At the phylum level, Actinobacteria was the most abundant in both the BS (37.8%) and MC (48.6%) samples, followed by Proteobacteria (mean values of 29.2% and 33.2%, respectively) and Firmicutes (mean values of 16.6% and 10.3%, respectively). Proteobacteria was the most abundant taxon (mean values of 34.9%) in the BT samples, followed by Actinobacteria (mean values of 26.4%) and Firmicutes (mean values of 23.0%). The phyla in the MX samples were Actinobacteria (mean 51.8%), Firmicutes (mean 25.8%), and Proteobacteria (mean 13.4%). The top three phyla were the same in each city subway station sample. The predominant bacterial phyla, Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria in our study, were also found on the subway platforms of New York City [57], Hong Kong [52], Barcelona [53], and Oslo [5].

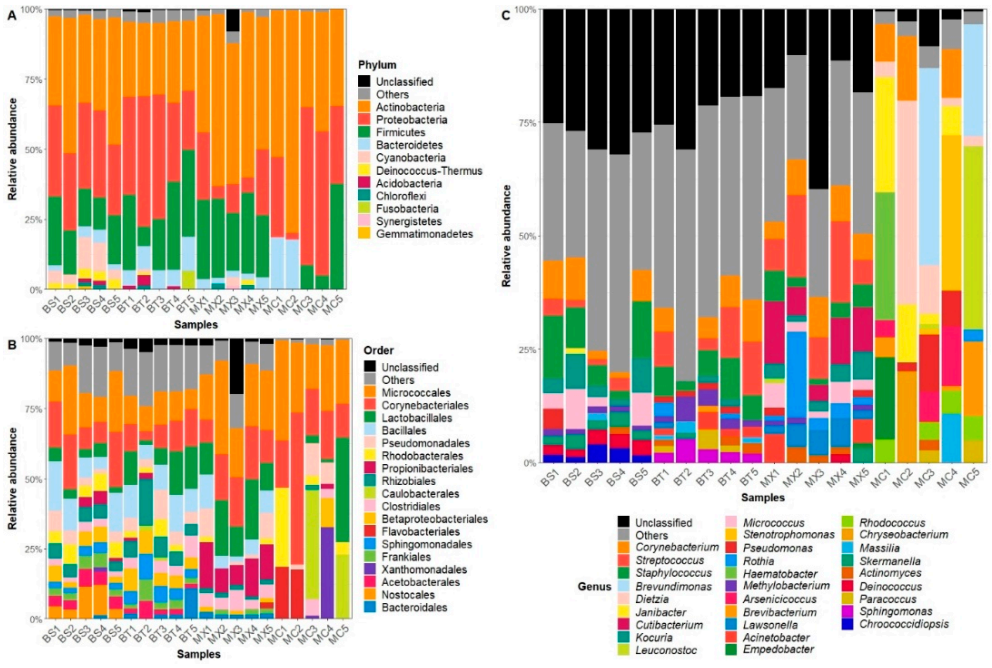

Figure 2. Relative abundance of major bacteria in BS, BT, MX, and MC samples (%) at the (**A**) phylum, (**B**) order, and (**C**) genus levels. Taxa with a relative abundance of ≥1% are indicated.

At the order level (Figure 2B), Micrococcales was the most abundant taxon (mean 17.0%), followed by Corynebacteriales (mean 14.4%), Lactobacillales (mean 8.7%) and Bacillales (mean 6.2%). Micrococcales (mean 14.5%), Corynebacteriales (mean 13.1%), Bacillales (mean 10.2%) and Nostocales (mean 6.1%) were prevalent in the BS samples, whereas Lactobacillales (9.0%), Micrococcales (mean 8.6%), Bacillales (mean 8.5%) and Corynebacteriales (mean 8.0%) were found in the BT samples. The abundant bacterial order in the MX samples were Micrococcales (mean 20.6%), Lactobacillales (mean 13.8%), Corynebacteriales (mean 13.5%) and Corynebacteriales (mean 10.8%). Micrococcales (mean 24.4%) was a

major bacterial order present in the MC samples, followed by Corynebacteriales (mean 23.2%), Caulobacterales (mean 12.2%), and Lactobacillales (mean 8.3%). Micrococcales and Corynebacteriales were both in the top 4 of the order level in each sample. Comparing the order that appeared in the top 5 in each sample, Lactobacillales had a considerably lower abundance showing in the BS samples (tenth, 3.5%), and Bacillales were not found in the MC samples.

The result of the genus composition demonstrated that the surface communities were similar despite the different sampling sites and regions (Figure 2C). In the subway microbiome of four countries, the three most abundant genera were *Corynebacterium* (5.55%), *Streptococcus* (4.65%), and *Staphylococcus* (4.14%). The top 5 genera in the BS samples were *Staphylococcus* (mean 7.6%), *Corynebacterium* (mean 5.0%), *Kocuria* (mean 4.1%), *Micrococcus* (mean 3.8%), and *Deinococcus* (mean 2.5%). In the BT samples, *Streptococcus* (mean 5.9%) was the most abundant genus, followed by *Staphylococcus* (mean 5.3%), *Corynebacterium* (mean 4.6%), *Methylobacterium* (mean 2.6%), and *Sphingomonas* (mean 2.6%). *Streptococcus* (mean 11.0%) was the most abundant genus in the MX samples, followed by *Cutibacterium* (mean 8.4%), *Corynebacterium* (mean 6.5%), *Rothia* (mean 5.3%) and *Lawsonella* (mean 4.0%). In contrast, *Brevundimonas* (12.2%), *Dietzia* (mean 11.2%), *Janibacter* (mean 8.9%), *Leuconostoc* (mean 7.7%), and *Stenotrophomonas* (mean 6.7%) were found in the MC samples.

Corynebacterium was the most abundant in all samples. Comparing the results of genus bacterial communities in the Oslo subway study in which both surface and air were sampled, *Corynebacterium* was found to be higher in surface samples than in air samples [5]. The subway station surface samples were dominated by Gram-positive bacteria, including genera *Corynebacterium*, *Streptococcus*, and *Staphylococcus,* and these results were consistent with previous studies [58,59]. Generally, Gram-positive bacteria have higher resistant characteristics than Gram-negative bacteria due to their spore-foaming survivability [58,59]. Previous studies have reported that *Staphylococcus* is positively associated with indoor temperature and humidity [60–62]. Temperature and humidity are the most obvious factors directly influencing the microbiome, but factors such as population density and ventilation systems are becoming increasingly important. Among them, existing studies have shown that the composition of the subway microbiome is strongly influenced by humans and provides an ideal environment for microbial propagation [63,64]. Oral-related microbial *Streptococcus* was positively correlated with temperature [65,66]. *Staphylococcus* and *Streptococcus* were not found in the MC samples, which had the lowest temperature and humidity. Moscow, a high latitude and cold region, has a different climate from other cities, the big differences of the atmospheric or meteorological factors between indoor and outdoor air may have influenced the microbial community differences [67,68].

Notably, in contrast with the BT and MX samples, the BS sample showed a higher percentage of *Staphylococcus* than that of *Streptococcus*. BS samples were sampled more recently than the two other cities. Due to COVID-19 at the time of sampling, wearing a mask, using hand sanitizers, and disinfecting subway stations were mandatory. Although *Staphylococcus* and *Streptococcus* are usually described together, their resistance to disinfectants is slightly different. *Staphylococcus* has been reported to be resistant to various disinfectants used to disinfect COVID-19 [69,70], but only a few studies on the resistance of *Streptococcus* to hydrogen peroxide have been reported [69]. Therefore, BS could be less *Streptococcus* enriched than BT and MX because of the impact of differences in hygiene practices on the microbiome of subway station spaces [64]. *Methylobacterium* genera has a capable of decomposing TVOC [71] and they were predominant in Busan, Boston and Mexico City. Previous study reported that TVOC and temperature significantly correlated in indoor Korea subway station [72]. In addition, *Sphingomonas* genera showed a positive correlation with temperature and PM values and a negative correlation with relative humidity [73,74]. The dominance of *Sphingomonas* genera in three cities subway samples may suggest that Moscow city has unique climate forms different microbial communities. However, the different tendencies of microbes in each city subway station may be caused by complex interactions among various factors, such as human skin, atmospheric environmental factors,

and passenger transport. Furthermore, we have yet to identify the relationship between human behavior and the subway microbiome.

Generally, human occupancy in indoor spaces is regarded as one of the primary sources of resuspending dust and releasing biomass [75,76]. *Corynebacterium*, *Streptococcus*, *Staphylococcus* and *Kocuria* are known as skin-associated microbiota [6,16,19,77]. *Cutibacterium* is a Gram-positive which is usually found in human skin and has a high abundance in moist or dry skin areas [78,79]. According to existing subway studies, one of the main sources of the subway microbiome is the skin microbiome, and the dominant microbiome change depending on the oily, moist, and dry degree and area of the skin [64]. *Stenotrophomonas* (6.7%) genera commonly found in soil were abundant in the Moscow subway station and were also investigated as the dominant bacteria in the New York subway [16,17]. In addition, *Dietzia* genera may be a human skin symbiosis and cause infections in humans [80,81]. Generally, microbial community of the subway station is not only of human origin but also has a large proportion of natural origins, such as soil and water [5,6,20]. For example, *Staphylococcus* and *Kocuria* genera commonly described human origin, while they are also known their origin was soil environments [5,6,20]. *Deinococcus* is mainly found in surface and air samples and is known to grow well over a wide temperature range from room temperature [82]. The genus *Roseomonas*, *Micrococcus*, *Methylobacterium*, and *Pseudomonas* commonly inhabit various terrestrial, aquatic, and atmospheric environmental samples, such as leaf surfaces, soil, dust, marine water, sand, vegetation, and freshwater [83–85]. *Blastococcus* is found in many places, including in the sea, soil, and vegetation, but has been investigated as being widespread mainly inside stones [86]. *Blastococcus* is also found at buildings such as limestone or marble stone surfaces [87], and by its nature it is not unusual to find many in subway stations. *Arsenicicoccus* found in other metro stations in Moscow from another study is not an etiological pathogen [27]. *Paracoccus* and *Sphingomonas* are soilborne microorganisms that were found in the Athens Metro, showing entry of outdoor soil material and road dust into subway stations [6]. *Chroococcidiopsis*, found in large numbers in BS samples, is mainly found in harsh natural environments [19]. These genera are less sensitive to external stresses, such as desiccation and radiation, and may be more favorable to environmental changes caused by the significant traffics in subway stations.

3.3. Surface Bacterial Community Structure

Compared with the bacterial community results of previous subway bioaerosol papers, the aerosol and surface microbial communities of the subway systems of major cities in the world are composed quite similar (Figures 1 and 2). In addition, the surface bacterial community shows that the human source tends to dominate and shares a bacterial community quite similar to that of air, indicating that microorganisms in the air can be attached and deposited on the surface [5,88]. As shown in PCoA (Figure 3), the bacterial compositions of all samples were clustered by sampling city, indicating a high degree of similarity (ANOSIM, statistic $R > 0.5$). Our samples were collected from various locations, such as door gates, touch screens, handle, ticket office, vending machine, floor, and lockers, with similar materials within the range of human activity. However, there were no significant differences between microbial communities between samples within the same city regardless of the sampling location ($p > 0.05$). This result is consistently shown in Mexico City, Boston, and Moscow samples, and insignificant differences in microbial communities between cities have been reported in previous studies [18–20,27]. Thus, what is particularly noteworthy in the PCoA results is that the three cities of Busan, Mexico City, and Boston clearly clustered among themselves, excluding Moscow, regardless of the sampling country. The microbial communities of the three regions show strong similarities in the grouping. In addition, PERMANOVA showed significant differences in the microbial community composition in each city ($p < 0.05$). Among the Busan samples, the BS1, BS2, and BS5 samples clustered close to Mexico City, while the BS3 and BS4 samples were similar to BT2 from Boston. This result suggested that the microbial community of the Busan subway samples seems to core microbiome between the Mexico City and Boston subway communities. The Moscow

samples were clearly distinct from the other three regions, and the distances between each sample within Moscow were far. These suggested that the environmental characteristics of the subway station, such as temperature, humidity, and the number of commuters, rather than the sampling location, may significantly affect the composition and structure of the bacterial community on the surface [2,4–6].

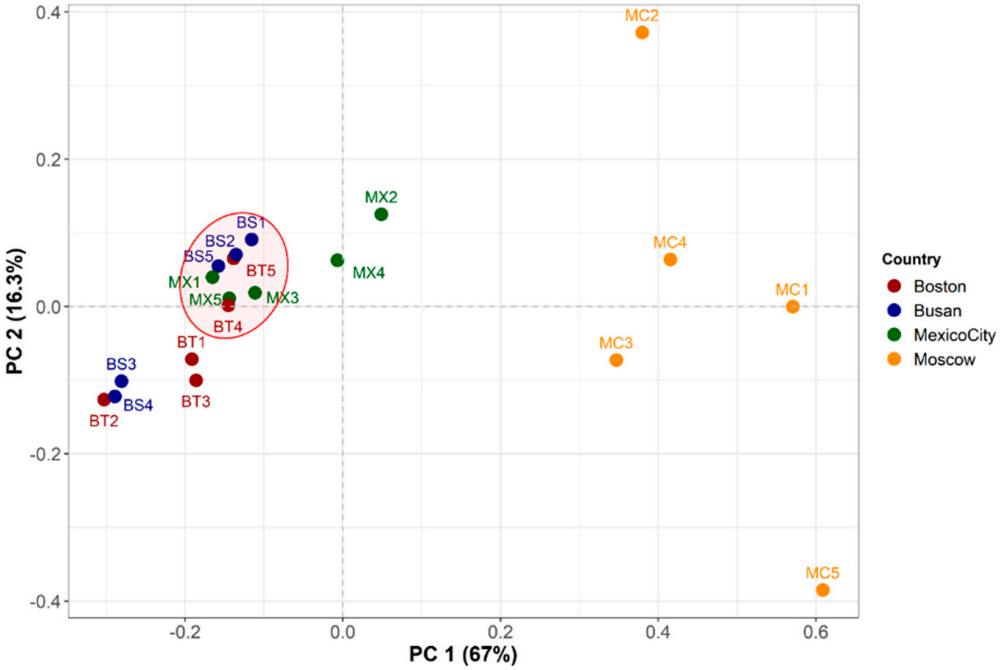

Figure 3. Principal coordinate analysis (PCoA) plot of bacterial compositions in global subway samples. Blue: Busan, Red: Boston, Green: Mexico City, Orange: Moscow.

According to Upset plot results, the number of identified genera was in the order of Boston, Busan, Mexico City, and Moscow (Figure 4). All samples shared 75 genera, while three cities, Busan, Boston, and Mexico City shared the most genus with 486 (Figure 4). As shown at the alpha diversity (Figure 1) and beta diversity (Figure 3), the uniqueness of Moscow microbial community is well represented in the upset plot at the genus level. The number of identified genus is around 1000 each in Busan and Mexico City, whereas only 100 in Moscow (Figure 4A). However, the small data set of the Moscow subway station genera is not unique to our study. An existing study, which was the source of the Moscow subway sequence of our study, has already reported a poor genus count [16]. A study analyzed the surfaces of other stations in Moscow also identified only Genus near 100 [27]. Even though there are very few genera in Moscow compared to other samples, the number of genera with a relative abundance of 1% or more was the highest in the Moscow sample at 31 (Figure 4B). Cities except Moscow share many microbial genera at more than 1%, while Moscow does not share a whopping 21 of 74 Genus. In connection with the result Figure 4A, the uniqueness of the Moscow microbiome does not come from a microbial genera that does not exist in other cities, but rather is a result of specific microbial genera being exceptionally dominant compared to other cities. Busan, Boston, and Mexico City share a total of six genera, namely, *Staphylococcus*, *Streptococcus*, *Kocuria*, *Methylobacterium*, *Skermanella*, and *Blastococcus*, at more than 1%. *Streptococcus* and *Staphylococcus* are representatives of human-derived microorganisms. In studies conducted in each region in Eurasia, these microbial genera are common in all sites related to the human oral cavity [89].

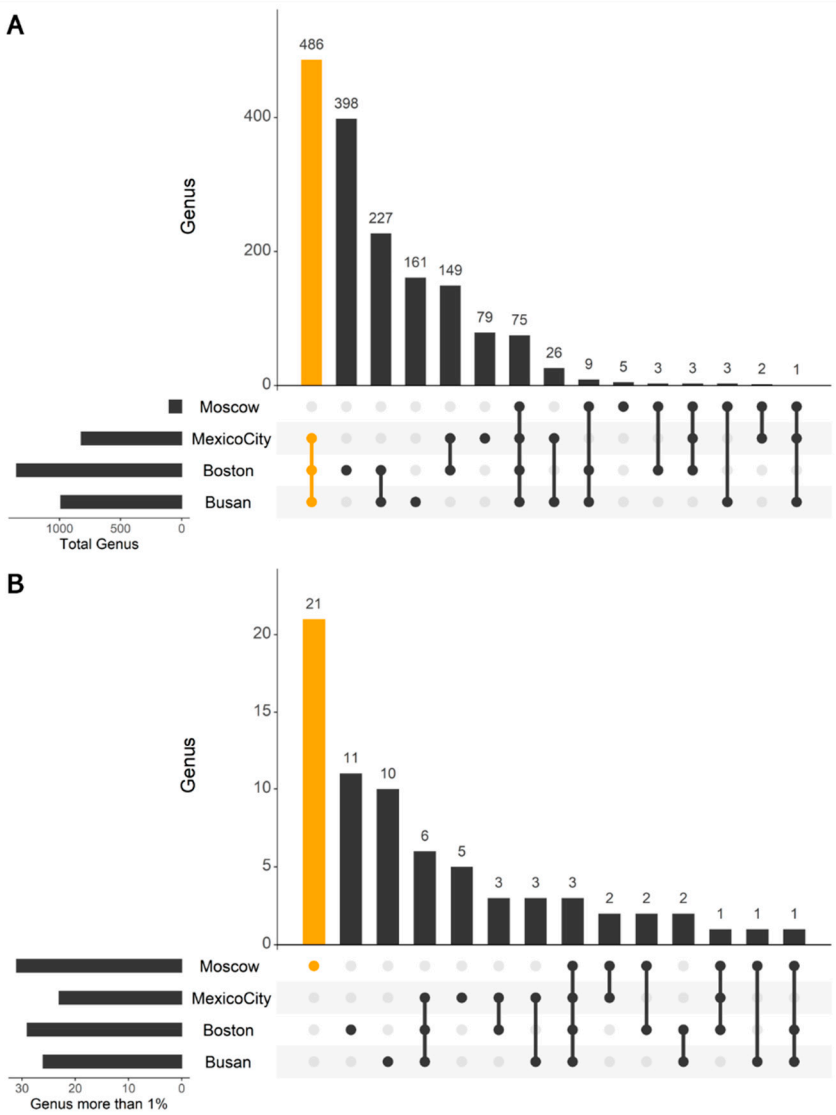

Figure 4. Upset plots of the bacterial genera among the cities. The plot was illustrated by (**A**) total genus and (**B**) genus more than 1%.

The swab method used in this study is widely accepted as the standard method for surface sampling [90–92]. The collection of airborne microbes, or bioaerosols, is the ideal way to directly assess the microbes to which humans are exposed. However, the collection of airborne microorganisms presents problems such as the sampling process being labor-intensive and expensive, so collecting sedimented surface samples is a more practical method [93]. In addition, airborne microbial sampling through vacuuming has the limitation of evaluating only transient changes, but surface samples may represent long-term exposed bacterial samples [88,94,95]. Millions of passengers use public transport systems daily, and commuters and passengers share the same air and surfaces. Since it is necessary to characterize the microbiome that humans contact with to evaluate the importance of indoor bacteria to human health, surface bacterial community can contribute to the open unknown indoor microbiome information.

4. Conclusions

Our study investigated the surface bacterial communities in subway stations in four large urban cities and compared them with those of other countries. We showed that the surface bacterial diversities and taxonomic profiling of the studied subway station surface environment were similar. Most of the predominant phylotypes were Gram-

positive microorganisms in all investigated samples and likely originated from human and outdoor sources. Although the ecological role of surface microbes remains unclear, our findings provided important insights into the structures of bacterial communities in different types of subway facilities as well as the major habitats of these microbes in subway station environments. In future studies, larger sample sizes and quantitative assessment of pathogens may provide additional insights into the ecology of indoor microorganisms.

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